1637

In re application of	:	Andrew S. Goldsborough
App. No.	:	10/031,636
Filed	:	April 29, 2002
For	:	ISOLATION OF NUCLEIC ACID
Examiner	:	Shar S. Hashemi
Art Unit	:	1637

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Cyclops Genome Sciences Limited
30 Pilgrim's Lane
London NW3 1SN

Patents ADP number *(if you know it)* 07651862001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention POLYNUCLEOTIDES

5. Name of your agent *(if you have one)* — Page White & Farrer

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POLYNUCLEOTIDES

Field of the Invention

The present invention relates to polynucleotides, modification of ribonucleic acid (RNA) to form oligo- and polynucleotides, and uses of such oligo- and polynucleotides.

Background to the Invention

RNA serves as an essential component of every modern biological study. It provides a raw material for medical diagnostics, drug design, recombinant protein production, bioinformatics and almost every area concerning the pharmaceutical and biotechnology industries.

RNA is an essential and universal component of all organisms. There are three major types of RNA; these are messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), the latter being the most common type. In addition, some viruses encode their genes in the form of RNA such as the retroviruses, HIV being one example of this type. RNA has many diverse functions such as in the production of proteins and the storage of genetic information. The ability of RNA to carry out these functions is dependent on its composition and sequence.

mRNA is naturally produced from a DNA template by a process known as transcription. It accounts for less than 5% of the total RNA in a cell and exists in hundreds of thousands of forms depending on its sequence; however, nearly all eukaryotic mRNA have a 5' CAP structure and a 3' poly $(A)^+$ tail, the latter serving as an essential feature for

purifying mRNA from the bulk of the cellular RNA. There are estimated to be 500,000 mRNA molecules in an average mammalian cell. It contains the coding region for a protein and is crucial to understand the function of a gene.

All RNA molecules are linear macromolecules composed of repeated monomers (ribonucleotides) comprising a base, a ribose sugar and a phosphate. There are four principal bases: uracil, cytosine, guanine and adenine; the order in which they are connected together, the sequence, leads to many of the unique properties of RNA.

RNA differs chemically from DNA in two major ways. Firstly, it contains uracil instead of thymine, and secondly, RNA has a 2'-OH group on the ribose sugar instead of 2'-H found on the deoxyribose sugar of DNA (see Figure 1a).

Natural RNA has the 2' carbon atom bonded to two other carbon atoms (C1' and C3'), a hydrogen atom and an oxygen atom that forms part of a hydroxyl group (here called the 2'-OH group). The 2'-OH group endows RNA with many of its unique properties such as structure, reactivity and instability. The 2'-OH group can also assist in the cleavage of the phosphodiester bonds between ribonucleotides leading to chain cleavage and hence RNA degradation (see Figure 1b).

When RNA is manipulated for any number of common laboratory practices, its inherent instability leads to considerable technical and experimental difficulties. For example,

measuring the abundance and size of a particular mRNA species is frequently considered essential to understanding the function of a gene. When the particular mRNA under study is degraded, even to a small extent, such measurements become impossible to carry out reliably or accurately. Another example would be the synthesis of a cDNA copy of a mRNA, where degradation of the mRNA precludes any possibility of obtaining a full and representative cDNA. Such cDNA copies are considered essential experimental tools because they allow a full and accurate characterisation of the gene such as its pattern of expression and chromosomal location. Furthermore the cDNA is essential to produce recombinant protein.

There are two principal means by which the 2'-OH group of ribonucleotides can be modified (a) enzymatically and (b) chemically. Enzymatic modification of the 2'-OH group arises from highly specific enzyme-catalysed reactions. For example, ribonucleotide reductase modifies the monomer ribonucleoside diphosphate, whereas an entire RNA molecule will not be recognised as a substrate. Another example is the methyl transferases that use an entire RNA molecule as a substrate but modify only a few 2'-OH groups per molecule.

The chemical synthesis of RNA and DNA is well known and many commercial services provide custom RNA and DNA syntheses (for review, see Eaton, (1995) *Annu. Rev. Biochem.* 64, 837). A considerable body of published work exists describing the different approaches to its synthesis (for review, see: Usman and Cedergreen (1992) *TIBS* 17:334) The most prominent route for preparation of 2'-modified

ribopyrimidines is through the introduction of nucleophiles to the corresponding 2,2'-anhydropyrimidine precursor. This reaction is limited to preparation of 2'-halides, 2'-azide, 2'-thiolates (Moffatt, (1979) In: *Nucleoside Analogues*, Ed. Walker, pp.71-163, NY, Plenum., Townsend, (1988) *Chemistry of Nucleosides and Nucleotides*, pp.59-67, NY, Plenum), 2'-azido (Verheyden, et al., (1971) *J. Org. Chem.* 36:250) and 2'-amino ribonucleoside (Wagner, et al., (1972) *J. Org. Chem.* 37:1876). Methylation of the 3', 5'-protected precursor gives 2'-*O*-methyl ribonucleosides (Sproat, et al., (1991) *Oligonucleotides and Analogues: A Practical Approach*, ed. F. Eckstein, pp.49-86, NY. Oxford Univ. Press), and similarly 2'-*O*-alkyl and 2'-*O*-allyl derivatives have been made (Sproat, (1991) *Nucleic Acids Res.* 19:733, Lesnik, et al., (1993) *Biochemistry.* 32, 7832). Other modifications include 2'-methyl (Matsuda, et al., (1991) *J. Med. Chem.* 34:234), 2'-phenyl, 2'-alkyl ribonucleosides (Schmit (1994) *Synlett.* 234), 2'-acetylated (Imazawa, et al., (1979) *J. Org. Chem.* 44:2039), 2'-fluoro, 2'-trifluoromethyl (Schmit, (1994) *Synlett.* 241), 2'-mercapto (Imazawa, et al., (1975) *Chem. Pharm. Bull.* 23:604) and 2'-thio ribonucleosides (Divakar, et al., (1990) *J. Chem. Soc. Perkin Trans.* 1:969). 2'-Fluoro, 2'-*O*-methyl, 2'-*O*-propyl and 2'-*O*-pentyl nucleotides have each been incorporated into oligoribonucleotides (Cummins, (1995) *Nucleic Acid Res.* 23:2019). In each case the substrates and products are non-polymerised, that is they exist as simple monomers and not in the polyribonucleotide (RNA) form.

Practical applications of such 2'-modified ribonucleotides and polyribonucleotides include anti-viral activity

(Wohlrab, et al., (1985) *Biochem. Biophys. Acta* 824:233), inhibition of bacterial growth (Salowe, et al., (1987) *Biochem.* 26:3408) and antisense oligonucleotides (Pieken, et al., (1991) *Science* 253:314). It has been shown that 2'-*O*-methoxyethyl replacement of the 2'-OH group can provide favourable conformations to enhance its binding to a target RNA. Research applications include developing novel ligands by the SELEX (systematic evolution of ligands by exponential enrichment) procedure (Gold, et al., (1995) *Annu. Rev. Biochem.* 64:763) and ribozyme research (Uhlenbeck, et al., (1987) *Nature* 328:596). The modification of the 2'-OH group as an investigative tool has been reviewed (Heidenreich, (1993) *FASEB J.*, 7:90). Many of the 2'-modified ribonucleotide triphosphates (Amersham International, Buckinghamshire, UK) or polymers (Midland Certified Reagent Company, Texas, USA) are available commercially.

Procedures suitable for modifying the 5'-OH and 3'-OH groups of deoxyribose have been developed in order to facilitate DNA oligonucleotide synthesis. For example, acetic anhydride in the presence of *N*-methylimidazole and tetrahydrofuran composes what is called the 'capping' reagent used commonly in almost all automated DNA synthesisers today. Other applications for acetic anhydride have been found, for example in the production of L-nucleoside dimers (Weis, *International Patent Application*, WO 97/11087).

Acetic anhydride in the presence of tetraethylammonium acetate has been used to produce synthetically short polyribonucleotides from 2'-acetylated monomers; the

product becomes completely resistant to pancreatic ribonuclease, an enzyme that requires the 2'-OH group in order to function efficiently (Rammler, et al., (1963) *J. Am. Chem. Soc.* 85:1989). Consequent treatment of such acetylated polyribonucleotides with ammonium hydroxide removes the acetyl function to restore the original 2'-OH group. Such RNA is degraded by ribonucleases, demonstrating that acetylation is fully reversible (Lapidot and Khorana, (1963) *J. Biol. Chem.* 85:3852).

Esterification at the 3' position with acetic anhydride was used to prepare poly(2'-azido-2'-deoxyuridylic acid) involving ammonium hydroxide for deacetylation (Torrence, (1972) *J. Amer. Chem. Soc.* 94:3638-3639). Pyridine-catalysed quantitative examples of acetylation are reported for 3'-hydroxynucleotides (Weber and Khorana, (1972) *J. Mol. Biol.* 72:219; Zhdanov and Zhenodarova, (1975) *Synthesis* 222).

The acetylation procedure was first described by Khorana and co-workers (Stuart and Khorana (1963) *J. Biol. Chem.* 85:2346) who acetylated the terminal 3'-OH group of deoxyribonucleotides and oligonucleotides with acetic anhydride. No modification of the bases was observed unless the acetylation was carried out in the presence of strongly basic solvents such as pyridine or tributylamine (Michelson and Grunberg-Manago, (1964) *Biochem. Biophys. Acta*, 91:92).

Acetylation of a tRNA molecule was carried out by using acetic anhydride. A change in the secondary structure was reported (Knorre, et al., (1965) *Biokhimiya* 30:1218).

Modification of 30% of the 2'-OH groups of tRNA was found to destroy its secondary structure. Further work by the same researchers demonstrated that variable acetylation levels of tRNA (Knorre, et al., (1966) *Biokhimya* 31:1181) and polyribo-oligonucleotides (Knorre, et al., (1967) *Biochim. Biophys. Acta* 142:555) could be achieved by use of acetic anhydride and *N,N*-dimethylformamide. It was also shown that acetylated poly(U) lost its ability to hydrogen bond with poly(A). Acetylated forms of poly(U) and poly(A) were reportedly quite unable to direct polypeptide synthesis in a cell-free system (Knorre, et al., (1967) *Molekul. Biol.* 1:837).

More recently, it has been reported in a publication that mRNA from a cell-free transcription system has been used as a substrate for acetylation (Ovodov and Alakhov, (1990) *FEBS* 270:111). Acetylation of 70-75% of the 2'-OH groups was said to be achieved using the method of Knorre et al. However, results presented in the publication suggest otherwise. Figure 2 shows no change in molecular weight indicating that no modification actually took place.

Summary of the Invention

In a first aspect, the present invention provides a polynucleotide comprising mRNA or viral RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position. The invention does not extend to polynucleotides consisting only of DNA or uses of oligo- or polynucleotides consisting only of DNA.

In a second aspect, the present invention provides a process for producing a modified oligo- or poly-nucleotide,

which comprises (i) contacting in a reaction medium RNA comprising an oligo- or poly-ribonucleotide with a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA; (ii) reacting the RNA with the reactant to produce modified oligo- or poly-nucleotide under conditions to achieve covalent modification of greater than 75% of the 2'-OH positions of the ribose rings; and (iii) optionally separating the modified oligo- or polynucleotide from the reaction medium, wherein the reaction medium comprises at least 20% v/v organic solvent. The RNA may be mRNA, tRNA, rRNA, viral RNA, synthetic RNA such as chemically synthesised or *in vitro* transcribed forms, or any other form of RNA, such as hnRNA. The RNA may be a mixture of different types of RNA and may be in single- or double-stranded form and even contain internal regions of secondary structure such as is commonly found in tRNA. According to the present invention an oligonucleotide generally has a sequence of up to about 80 bases and a polynucleotide generally has a sequence length of more than about 80, preferably more than about 100 bases. A preferred length for a polynucleotide is at least 1kb.

The mRNA may or may not have a cap and/or polyA tail. The mRNA or viral RNA used in the present invention is preferably naturally-occurring. A naturally-occurring RNA according to the present invention typically comprises a nucleotide sequence which is found in nature and which generally encodes a polypeptide having biological activity, or such a nucleotide sequence which is modified, for example to alter in some way the biological activity of the polypeptide encoded thereby. Whilst the naturally-

occurring RNA is preferably obtained by transcription from a suitable template, itself usually naturally-occurring, in some cases the naturally-occurring RNA can be obtained synthetically. mRNA according to the present invention does not encompass simple homopolynucleotides (polyA, polyU, polyG and polyC) which can be generated synthetically but are biologically non-functional.

As described in further detail below, other steps in the process may include (*iv*) using the modified RNA as a template in order to produce a second complementary strand of RNA or DNA, and (*v*) ligating suitable DNA fragments such as a plasmid vector to the ends of the molecule in order to clone and propagate it. An important aspect of this invention is modification of mRNA since it is of major scientific interest and serves as a good example of the problems encountered when manipulating RNA. The invention further provides methods for obtaining intact full-length copies of mRNA and other types of RNA isolated from cellular sources that demonstrate increased stability in conditions that would otherwise destroy a major fraction of the unmodified RNA.

Modification at the 2'-OH position is preferably substantially regiospecific. Thus, there is preferably substantially no modification of the bases, phosphodiester bonds and/or any other position within the RNA chain. In this way, the polynucleotide retains important properties of the RNA. For example, advantageously, the polynucleotide is preferably modified so that a single strand of the polynucleotide is replicable by a nucleic

acid polymerase to generate a second strand of polynucleotide complementary to the single strand.

The extent of modification of the 2'-OH position of the ribose rings should be sufficient to protect the polynucleotide against nuclease degradation, especially against cellular endonucleases and/or intracellular concentrations of nucleases. Preferably at least 80%, more preferably 85% of the ribose rings are covalently modified at the 2'-OH position, still more preferably at least 90% and most preferably at least 95% of the ribose rings are covalently modified at the 2'-OH position.

The modification at the 2'-OH position may be such that the entire OH of the 2'C of the ribose ring is replaced by a reactant group R as in 2'-R or by OR having 2'-OR where the -O- group may or may not originate from the 2'-OH group. Accordingly, the substituent at the 2'-OH position in this case is R or OR respectively. An aim of the modification is to protect the molecule to a significant extent from degradation. Degradation may be a result of nucleases, metal ions and/or high temperatures, high pH or other chemical or physical conditions.

It will be apparent to those skilled in the art that multiple types of substituents exist which are suitable to practice this invention. One set of examples is given here for clarity for the acyl group - COR substituent where R can be composed solely of carbon, oxygen and hydrogen atoms in a linear chain arrangement, as in $-COCH_2CH_2CH_2CH_3$, in a branched chain arrangement as in $-COC(CH_3)_3$ or in a ring

structure as in benzoate - Bz. It will be further understood that hydrogen can be replaced by other atoms as in $-COCH_2Cl$ or $-COCF_3$ and that carbon atoms can be joined to another carbon with one or more bonds as in the crotonate $-COCH_2CH=CHCH_3$ or one or more oxygen atoms as in the ether $-COCH_2CH_2OCH_3$ or a combination of both as in the crotonate ether - $COH=CHCH_2OCH_3$. Furthermore, other atoms such as nitrogen, silicon and sulphur may also be present. A single RNA molecule may bear more than one type of substituent on any of its 2'-OH positions producing mixed substituent RNA chains.

The modified ribose rings may bear at the 2'-OH position a variety of substituents. The substituent may have the formula OR, wherein R is selected from C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, C_1-C_{10}, alkoxyalkyl, C_1-C_{10} haloalkoxyalkyl, C_1-C_{10} aminoalkoxyalkyl, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl, C_6-C_{14} arylalkenyl, C_1-C_{10} alkanoyl, C_1-C_{10} alkenoyl, C_1-C_{10} haloalkanoyl, C_1-C_{10} aminoalkanoyl, C_6-C_{14} arylalkanoyl, C_6-C_{14} arylalkenoyl, C_6-C_{14} aryloxyalkanoyl, C_6-C_{14} alkylarylalkanoyl, C_6-C_{14} haloarylalkanoyl, C_6-C_{14} aminoarylalkanoyl, C_1-C_{10} alkylsilanyl, C_{12}-C_{28} diarylphosphone. In this case R is preferably selected from methyl, ethyl, vinyl, allyl, ethynyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, (2-chloroethyl)oxyethyl, (2-aminoethyl)oxyethyl, phenyl, 4-methylphenyl, benzyl, cinnamyl, acetyl, propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl, octanoyl, pivaloyl, 4-aminobutanoyl, 4-chlorobutanoyl, trifluoroacetyl, trichloroacetyl, acryloyl, propioloyl, crotonoyl, benzoyl, diphenylacetyl, phenoxyacetyl, 4-methylbenzoyl, 4-chlorobenzoyl, 4-

aminobenzoyl, 4-nitrobenzoyl, cinnamoyl, silanyl, trimethylsilanyl, t-butyldimethylsilanyl, 2-chlorophenyl(4-nitrophenyl)phosphono. Alternatively, the substituent may be R', wherein R' is selected from C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, halo, amino, C_1-C_{10} alkylamino, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl. In this case the R' is preferably selected from methyl, ethyl, vinyl, allyl, ethynyl, t-butyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, phenyl, benzyl, fluoro, chloro, amino, keto.

Various reactants or reactant combinations may be used, optionally in the presence of a catalyst, to provide these substituents, as described in further detail in the Examples below. Advantageously, the reactant comprises an acid anhydride, an acid chloride, a carboxylic acid or an N-acylimidazole, each of these reactants participating in an acylation reaction with the RNA. Under these reaction conditions, the reaction medium may further comprise an acylation catalyst. For example, where the reactant comprises an acid anhyride, this may be reacted with the RNA in the presence of a catalyst such as a fluoride ion or amino pyridine. As a further example, where the reactant comprises an acid chloride or N-acylimidazole, the reactant may be reacted with the RNA in the presence of an amino pyridine. As a further example, where the reactant comprises a carboxylic acid, this may be reacted with the RNA in the presence of a dehydrating agent or a catalyst, such as an isocyanide catalyst. A preferred aminopridine catalyst is dimethyl aminopyridine (DMAP).

The organic solvent used in the reaction medium of the present invention preferably comprises an organic base and may comprise an organic solvent in which is dissolved the organic base or, in a preferred embodiment, may be the organic base itself. It is preferred that the reactant is soluble in the organic solvent. In a preferred embodiment the reaction medium further comprises water. In this way RNA to be modified may be conveniently added to the organic solvent as an aqueous solution of RNA. Typical organic solvents include alkanes such as hexane and pentane, pyridine, acetonitrile, dimethylformamide, dichloromethane, acetone, diethyl ether, benzene, chloroform, ethyl acetate, light petroleum, tetrahydrofuran, carbon tetrachloride, dichloroethane, dioxane, carbon disulphide, nitromethane, dimethyl sulphoxide, hexamethylphosphoric triamide and toluene. Typical organic bases include pyridine, triethylamine, trimethylamine, diisopropylethylamine, *N,N*-diethylaniline, *N,N*-dimethylaniline, 1,5-diazabicyclo (4,3,0) non-5-ene (DBN), 1,8-diazabicyclo (5,4,0) undec-7-ene (DBU) and *N*-methylmorpholine. Triethylamine $(CH_3CH_2)_3N$ is a stronger amine base than pyridine, aniline, diethylamine or trimethylamine but less so than pyrrolidone. It is one of the strongest amine bases. A preferred organic base which acts as a solvent is triethylamine (TEA). Where a catalyst is to be used, it is convenient for the catalyst to be soluble in the organic solvent as well. The water and the organic solvent may form different phases in the reaction medium. For example, the water and the organic solvent may be immiscible with one another and form phases which will separate upon standing. Where there is more than one phase, the RNA may

be reacted with the reactant under conditions of phase transfer catalysis.

The amounts of water and organic solvent may be varied and will depend to some extent upon the particular organic solvent/base/catalyst system to be used. Advantageously, the reaction medium comprises at least 50% organic solvent, preferably at least 80%, more preferably at least 90% and more preferably at least 95% v/v. Typically, the ratio of water:organic solvent is in the range 1:50 to 1:10, preferably around 1:20.

In the absence of a catalyst, the reaction time is generally from 20 to 60 mins. In the presence of the catalyst, the reaction proceeds more quickly, the reaction time generally being completed within about 20 seconds.

On a vol/vol basis it is found that the ratio of reactant to reaction medium (especially acetic anhydride triethylamine/DMAP) is preferably in the range 1:200 to 1:10, more preferably around 1:20. Too little reactant gives a partial reaction and too much makes the reaction difficult to control.

In certain circumstances, it may be advantageous prior to step (*i*) of the process of the present invention to use a step of protecting the exocyclic amino groups of the bases of the RNA with a protecting group. After step (*ii*) a step of deprotecting the exocyclic amino groups by removing the protecting group may be used. In this way, unwanted side-reactions between reactants and the exocyclic amino groups is avoided. For adenine, the protecting group may be

benzoyl, *N*-phenoacetyl or *N,N*- dimethylaminomethylene. For cytosine, the protecting group may be benzoyl. For guanine, the protecting group may be isobutyl, *N*-phenoacetyl or *N,N*-dimethylaminomethylene. Crown 18-6 has been found to be a useful protecting agent to protect the exocyclic primary amino group from acylation, essentially with acetic anhydride (Barrett & Lana, J.C.S. Chem. Commun. 471, 1978).

In one aspect, the RNA which is modified comprises an RNA sample from a cell extract. The RNA sample may be a total RNA sample or a purified RNA, such as an mRNA.

RNA is generally purified in order to study gene expression, determine the size and structure of the mRNA, identify gene products, determine its abundance and to clone it as a DNA copy. Purifying intact and complete copies of RNA is one of the first, critical steps in many molecular biology protocols yet it is also one of the most difficult to carry out successfully. Although there are any number of means by which to purify RNA, all extraction methods involve four steps: (1) inactivation of nucleases, (2) separation of RNA from proteins, (3) separation of the RNA from other macromolecules and (4) concentration of the RNA. To purify the mRNA fraction from the total RNA another step is involved, that is (5) separation of poly (A) tailed RNA from other types.

The choice of the purification system depends on a number of factors such as the source of RNA, its abundance and its ultimate use. One of the most important aspects when isolating RNA is to prevent any degradation during the process. All cells contain enzymes capable of destroying

mRNA called ribonucleases which must be removed or rapidly inactivated during the process of mRNA isolation. The ubiquitous nature of 'nuclease' is illustrated by their presence in secretions from finger tips and dust; contamination by any of these will inevitably lead to RNA degradation. Instability of RNA makes it very difficult to isolate it intact, since even a single break in the chain will make this impossible.

RNases (enzymes capable of degrading RNA) are notoriously difficult to inactivate because unlike DNases they do not require cofactors, are heat stable and refold rapidly following heat denaturation. Some tissues such as the pancreas and spleen contain particularly high concentrations of RNases. Unlike DNases, RNases do not require metal ions for activity and therefore cannot be inactivated by metal chelating substances such as EDTA. Some RNases can do without a metal ion for activity because they use the 2'-OH groups instead as a reactive species. Many RNases such as RNase A can survive autoclaving temperatures (120°C) because the polypeptide readily refolds to assume its original active structure on cooling. This is rarely a property of DNases which become permanently inactivated on heating at moderate temperatures such as 65°C. Due to the extreme difficulty of inactivating RNases, several harsh methods have been developed. These include the use of an alkylating agent such as diethyl pyrocarbonate (DEPC) which permanently modifies the active site of RNase A, or denaturing agent such as guanidinium isothiocyanate. DEPC unfortunately is a suspected carcinogen. Other commercially available RNase inhibitors include ribonucleoside vanadyl complex and

angiogenin-binding protein. The former reagent has limited use because it will inhibit the majority of enzymes and the latter is very expensive.

One of the most commonly used methods for purifying RNA are those based on Chirgwin et al., (1979) *Biochemistry* 18:5294-5299 and Chomczynski and Sacchi, (1987) *Anal. Biochem.* 162:156 and useful descriptions of how to correctly handle RNA can be found in Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual* (2nd Ed.) Cold Spring Harbor University Press, Cold Spring Harbor, NY. Many companies also provide RNA isolation kits such as MICRO FAST TRACK™ from Invitrogen, PolyATract® from Dynal, Norway and TRIzol Reagent™ from Gibco BRL of Gaithersburg, USA.

In one embodiment, at least some of the modified ribose rings bear at the 2'-OH position a substituent which is labelled with a label. Useful labels include fluorescent or radioactive labels as well as ligands for antibodies or other proteins, for example biotin, or specific types of metal ions such as tin. Various uses for labelled oligonucleotides or labelled polynucleotides are discussed below.

In a further aspect, the present invention provides a kit for modifying an oligo- or polynucleotide comprising an oligo- or poly-ribonucleotide, which kit comprises

(a) an organic solvent; and

(b) a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the oligo- or poly-ribonucleotide in the presence of the organic solvent. The kit may be used to modify an oligo- or polynucleotide which

conveniently comprises an aqueous sample. Alternatively, the oligo- or polynucleotide may be present in a non-aqueous solvent.

In a further embodiment there is provided a method for gene expression analysis which comprises obtaining a polynucleotide comprising an mRNA sample modified in accordance with the above process where the RNA sample is from a cell extract. The polynucleotide is analysed, for example, by hybridisation probing. Commonly-used methods of gene expression analysis include northern blotting, RT-PCR, dot blotting and *in situ* hybridisation. These methods require mRNA in an intact form capable of serving as a marker of gene expression. By modifying the 2'-OH group in accordance with the present invention, the extent of degradation of the mRNA is reduced.

In a further aspect, the present invention provides use of an oligo- or poly-nucleotide comprising RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position, as a probe. The probe may be labelled, for example, with a fluorescent or radioactive label. For example, modified mRNA may serve as a labelled probe for hybridisation, finding utility, for example, in "biochip" applications used to study gene expression.

Currently, an entire mRNA population is reverse transcribed in the presence of a radioactive deoxynucleotide triphosphate such as ^{32}P dATP to produce a labelled cDNA probe which is then hybridised to the 'biochip'. In this invention, as described in Example V, the modified mRNA itself serves as the probe. Probes prepared in this way

would have very high specific activities (cpm/μg RNA) and therefore be capable of detecting very small amounts of target DNA or RNA. Alternatively, fluorescent silyl groups could be used as labelling groups for RNA (Horner, et al., (1985) J. Organomet. Chem. 282:175).

In a further aspect, the present invention provides a method for the replication of a polynucleotide, which comprises obtaining a polynucleotide comprising modified RNA as described above, and replicating the modified RNA to form a complementary polynucleotide using a nucleic acid polymerase. Because modification of RNA in accordance with the present invention can provide a replicable polynucleotide which is relatively stable to laboratory manipulation, the polynucleotide may be used in a range of applications as a substitute for DNA. The complementary polynucleotide may comprise an RNA, DNA or hybrid or modified forms thereof.

For example, the complementary polynucleotide may comprise a cDNA and the nucleic acid polymerase may comprise a DNA polymerase. Such polymerases are discussed in detail below.

The copying of mRNA into cDNA is an important method for obtaining fully representative copies for use in applications including gene patenting, DNA sequencing, protein production for drug screening programs and understanding the function of a particular gene. Conventionally, all require the activity of reverse transcriptase which is associated with many associated problems such as inhibition.

The synthesis and cloning of cDNA involves a complex series of enzymatic steps in order to copy the mRNA into double-stranded DNA and cloning this into a DNA vector. As used herein the term cDNA refers to a complementary DNA molecule synthesised using a ribonucleic acid strand (RNA) as a template. Many approaches are known for cDNA cloning, all have tried to preserve as much of the original sequence as possible (Okayama and Berg, (1982) *Mol. Cell. Biol.* 2:161, Gubler and Hoffman, (1983) *Gene* 25:283).

Conventionally, problems can occur at one or more of three stages, 1) mRNA isolation, 2) first strand cDNA synthesis or 3) second strand synthesis. When the mRNA starting material is degraded, incomplete forms of the cDNA are an inevitable result. One application of the present invention is to stabilise the mRNA molecule in order to isolate complete copies of the mRNA. mRNA modified in accordance with the present invention can be used as a template for reverse transcriptase.

Obtaining a full length cDNA is one of the most difficult yet important tasks when characterising a gene. Most commonly, cDNA libraries are produced by the complete conversion of a mRNA pool into a cDNA copy (Gubler and Hoffman (1983) *Gene* 25:263-269) however the most common outcome is to produce an incomplete representation of the starting mRNA.

Methods to isolate full length cDNA copies of mRNA include: RACE (rapid amplification of cDNA ends) first described in 1988 as a method to isolate full length cDNA's using PCR (Frohmann, et al., (1988) *Proc. Natl. Acad. Sci.* USA 85,

8998-9002). Related methods have been reviewed (Schaefer, (1995) *Anal. Biochem.* 227:255-273). Although these methods can be successful for retrieving the 5' and 3' ends of single cDNA molecules, it requires considerable skill and depends in large part on the abundance of the mRNA and can only be done one at a time.

The method for the replication of the polynucleotide, according to the present invention, may further comprise a step of ligating to a vector a single- or double-stranded polynucleotide comprising the polynucleotide and the complementary polynucleotide. In this way, molecular cloning procedures may be accomplished using modified RNA according to the present invention.

In a further aspect, the present invention provides use of an oligo- or poly-nucleotide comprising RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position, as a template for polymerase chain reaction. This use avoids the need for the conventional step of copying mRNA into cDNA using reverse transcription (RT-PCR) so that the RNA serves as a template for DNA polymerases (PCR) such as the commonly used Taq polymerase (DNA amplification procedures are well known and are described in U.S. Pat. No. 4,683,202). The enzymatic reverse transcriptase (RT) stage is strongly influenced by reaction conditions such as salt concentration, buffer type, pH, temperature and primer. The enzyme reverse transcriptase is also known to be strongly inhibited by mRNA secondary structure, as well as creating chimeric cDNA molecules due to transfer of activity from one mRNA template to another. The limitations in sensitivity of

the PCR amplification is directly related to the quality of the reverse transcription step. Therefore to be able to avoid this problematic stage, modified mRNA may be used as a template for PCR. This would involve two steps, 1). chemical modification of the mRNA, 2) PCR amplification. RT-PCR is invaluable for detecting gene expression, for amplifying RNA sequences prior to cloning and analysis and for the diagnosis of infectious diseases and genetic diseases.

In a further aspect, the present invention provides, a method for producing a double-stranded oligo- or polynucleotide from a template, which comprises contacting the template with a plurality of mononucleotides comprising UTP, dTTP and/or dUTP, ATP and/or dATP, GTP and/or dGTP, and CTP and/or dCTP, in the presence of a nucleic acid polymerase and optionally a template primer under conditions to polymerise the mononucleotides to form a nucleic acid strand complementary to the template, wherein the template comprises an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH.

It has been surprisingly found that when the ribose rings of the oligo- or polyribonucleotide are modified in accordance with the present invention, the oligo- or polyribonucleotide produced thereby is capable of acting as a template for one or more of a variety of nucleic acid polymerases. Nucleic acid polymerases within the scope of the present invention include DNA polymerases, RNA dependent polymerases and RNA dependent RNA polymerases.

Among the RNA-dependent DNA polymerases are Superscript™ II (MMLV reverse transcriptase RNase H-), MMLV reverse transcriptase, HIV reverse transcriptase, AMV reverse transcriptase, RAV-2 reverse transcriptase, human T-cell leukemia virus type I (HTLV-I) reverse transciptase, bovine leukemia virus (BLV), Rous Sarcoma virus (RSV), Tth DNA polymerase, Tfl DNA polymerase, Bst polymerase, Taq DNA polymerase, Thermoscript, C.therm polymerase, displaythermo-RT or Klenow DNA polymerase.

Among the DNA-dependent DNA polymerases are DNA polymerase I;-Klenow fragment; T4 DNA polymerase; T7 DNA polymerase; Taq DNA polymerase, Tli DNA polymerase, Pfu DNA polymerase; Vent™ DNA polymerase; Deep Vent™ DNA polymerase; Bst DNA polymerase; Tth, Pfu Turbo™, Pfu(exo-), Pwo, Pyra™, Tfu, KlenTaq, Taq2000™, AmpliTaq Stoffel fragment, Sequenase™, Tma, Vent®(exo-), Deep Vent®(exo-) or a DNA polymerase purified from Thermosipho africanus, Thermotoga maritima, Desulfurococcus mobilis, Methanobacterium thermoautotrophicum, Methanothermus fervidus, Pyrococcus furious, Pyrodictium occultum, Sulfolobus acidocaldarius, S. solfataricus, Thermococcus litoralis or Thermoplasma acidophilum.

Among the RNA-dependent RNA polymerases are Q beta replicase, or is derived from E. coli phage f2, R17, MS-2 or ø6, or from a virus family selected from bromoviridae, flaviviridae, picornaviridae, potyviridae, tobamovirus, tombusviridae, leviviruses, hepatitis C-like viruses, and picornaviruses or from polio virus, yellow fever virus, tobacco mosaic virus, brome mosaic virus, influenza virus, reovirus, myxovirus, rhabdovirus and paramyxovirus.

Nucleic acid may be classified into four overlapping groups. Classification is based on the type of template copied (RNA or DNA) and the type of complementary nucleic acid strand that is produced (RNA or DNA), Although *in vivo*, nucleic acid polymerases have discrete activities, *in vitro* specificity for the template and the substrate mononucleotides is less stringent. As one example, *in vitro* certain DNA dependent DNA polymerases such as Taq and Tth DNA polymerase can also behave as RNA dependent DNA polymerases. Specificity depends in part on the buffer conditions, presence of metal ions and the type of mononucleotide triphosphate present. Lastly, many mutant forms of polymerases are known (for one example see; Gao et al., (1997) Proc. Natl. Acad. Sci (USA) 94:407) that are less specific with respect to the template strand copies and the type of complementary strand produced. Accordingly, some enzymes appear in more than one of the above lists.

Preferably, the oligo- or poly- nucleotide is modified by (i) contacting in a reaction medium RNA comprising an oligo- or poly-ribonucleotide with a reactant capable of covalently modifying the 2′-OH position of the ribose rings of the RNA; (ii) reacting the RNA with the reactant to produce modified oligo- or poly-nucleotide under conditions to achieve covalent modification of greater than 75% of the 2′-OH positions of the ribose rings; and (iii) optionally separating the modified oligo- or polynucleotide from the reaction medium, wherein the reaction medium comprises an organic solvent.

In a further aspect, the present invention provides a method for amplifying an oligo- or polynucleotide, which comprises:

(1) providing the oligo- or polynucleotide as a template comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH position;

(2) producing from the template a double-stranded oligo- or polynucleotide in accordance with the above method;

(3) melting each double-stranded oligo- or polynucleotide to form single strands;

(4) annealing the template primer to the single strand having the nucleotide sequence of the template and annealing a second primer to the strand complementary thereto to form primed single strands;

(5) contacting the primed single strands with the plurality of mononucleotides in the presence of the nucleic acid polymerase to form double-stranded oligo- or polynucleotides;

(6) optionally repeating steps (3) to (5) until sufficient amplification is achieved; and

(7) harvesting the amplified oligo- or polynucleotide in single- or double-stranded form.

This method is typically used in a polymerase chain reaction.

In a further aspect the present invention provides a method for amplifying an oligo- or polynucleotide, which comprises:

(1) providing the oligo- or polynucleotide as a template comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH position;

(2) amplifying the template in a nucleic acid sequence based amplification (NASBA), and

(3) harvesting the amplified oligo- or polynucleotide in single- or double-stranded form, wherein the step of amplifying the template including producing from the template a double-stranded oligo- or polynucleotide in accordance with the above method.

There is further provided a method for diagnosing in a subject a disease indicated by the presence or absence of a target nucleotide sequence, which method comprises:

(a) obtaining an oligo- or polynucleotide sample from the subject;

(b) amplifying the oligo- or polynucleotide in accordance with either of the above methods to form an amplified oligo- or polynucleotide; and

(c) analysing the amplified oligo- or polynucleotide for the target nucleotide sequence. The subject may be a human, an animal or a plant.

In a further aspect, the present invention provides use of a nucleic acid polymerase for the production of a nucleic strand complementary to a template for the nucleic acid polymerase, wherein the template comprises an oligo- or polynucleotide comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or

polyribonucleotide are covalently modified at the 2'-OH position.

The nucleic acid polymerase may be any of those nucleic acid polymerases defined above.

In a further aspect, the present invention provides use of an oligo- or polynucleotide comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH position, as a template for a nucleic acid polymerase.

Either of these uses relate to, for example, reverse transcription or use in a polymerase chain reaction, including RT-PCR.

In a further aspect, the present invention provides a kit for producing a nucleic acid strand complementary to an oligo- or polynucleotide comprising an oligo- or polyribonucleotide, which kit comprises:

(a) a nucleic acid polymerase;

(b) a reaction system for modifying the oligo- or poly-nucleotide to form a template for the nucleic acid polymerase in which greater than 75% of the ribose rings of the oligo- or poly-ribonucleotide are covalently modified at the 2'-OH position;

(c) optionally a plurality of mononucleotides comprising UTP, dTTP and/or dUTP, ATP and/or dATP, GTP and/or dGTP, and CTP and/or dCTP; and

(d) optionally a buffer for the nucleic acid polymerase.

Typically, the reaction system comprises:

(i) an organic solvent preferably comprising an organic base; and

(ii) a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the oligo- or poly-ribonucleotide in the presence of the organic solvent.

The kit may be used for a variety of applications including reverse transcription to produce, for example, full length cDNA, a kit for producing a template for SELEX, a kit for NASBA, a kit for producing a template for sequencing, or a target for hybridisation.

The kits optionally further comprise appropriate buffer systems depending on the use to which the kit is to be put and the specificity of the nucleic acid polymerase which is required.

In a further aspect, the present invention provides a method for replicating an oligo- or polynucleotide, which comprises:

(1) providing the oligo- or polynucleotide as a template comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH position;

(2) producing from the template a double-stranded oligo- or polynucleotide in accordance with the above method;

(3) ligating the double-stranded oligo- or polynucleotide into a vector; and

(4) replicating the vector in a host.

In a further aspect, the present invention provides a method for replicating an oligo- or polynucleotide, which comprises:

(1) providing the oligo- or polynucleotide as a template comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH position;

(2) ligating the template into a vector;

(3) producing from the template in the vector a double-stranded oligo-or polynucleotide in accordance with the above method; and

(4) replicating the vector in a host.

In a further aspect, the present invention provides a method for replicating an oligo- or polynucleotide, which comprises:

(1) providing the oligo- or polynucleotide as a template comprising an oligo- or polyribonucleotide, greater than 75% of the ribose rings of which oligo- or polyribonucleotide are covalently modified at the 2'-OH position;

(2) producing from the template a double-stranded oligo-or polynucleotide in accordance with the above method;

(3) obtaining from the double-stranded oligo- or polynucleotide the nucleic acid strand complementary to the template;

(4) ligating the nucleic acid strand into a vector; and

(5) replicating the vector in a host.

According to each of these methods, modified RNA according to the present invention may be used in a cloning procedure.

Although cloning of DNA is well known and commonly carried out (Sanbrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH), the following alterations may be expected to enhance cloning of modified RNA. In particular, the following alterations of the basic protocol would be expected to provide longer cDNA inserts.

The RNA modification reaction (example 6 and others) may also lead to the modification of the 5'-phosphate group of the RNA substrate in addition to the 3'-OH group and 2'-OH groups. In the case of mRNA which has a common 5'CAP structure it would be expected that the CAP is also modified. In order to allow cloning of the modified RNA into a vector it is necessary to remove both the CAP and the 3' terminal nucleotide.

Alternatively for RNA strands with no CAP structure direct removal of the modified 5'-phosphate group can be carried out either with shrimp or calf alkaline phosphatase, it has been found that acetylated RNA with a 5'-triphosphate structure, as is common to all synthetic RNA strands, can be dephosphorylated using shrimp alkaline phosphatase and rephosphorylated with T4 polynucleotide kinase (Sambrook et al., (1989) molecular Cloning: A Laboratory Manual, CSH).

The CAP structure is commonly removed by either an enzymatic (Jones et al.,(1994) in RNA Isolation and Analysis. Bios. Oxford p77) or chemical procedure (Stahl et

al., (1989) in *Nucleic Acids Sequencing: A Practical Approach.* IRL Press, Oxford p137). The 3' modified nucleotide can be removed by the brief exposure to 3' exonucleases such as a snake venom phosphodiesterase (Crotalus durissus). Alternatively the 3' exonuclease activities of T4 DNA polymerase or Klenow fragment DNA polymerase could be exploited.

To enhance the ligation of single stranded nucleic acids into either a single or double stranded DNA vector the following procedure may be used. T4 DNA ligase will not ligate single stranded nucleic acids, therefore a region of double stranded nucleic acid is produced at each end of the cloning site. The restriction enzyme Hga 1 produces a 5 nucleotide 5' overhang and BstX1 produces a 4 nucleotide 3' extension and these are used in conjunction with appropriate cloning vectors to produce ligation sites for the single stranded nucleic acid insert. Inserts can either be ligated with DNA ligase or alternatively with RNA ligase.

A double stranded form of the insert can be produced in two ways: firstly by transforming appropriate *E.coli* hosts and allowing the host polymerases to produce the second strand; and secondly *in vitro* by extending from the free 3'-OH group of the vector or oligonucleotide primers with enzymes such as AMV reverse transcriptase, T4 DNA polymerase, T7 DNA polymerase or Klenow fragment DNA polymerase. The addition of single stranded DNA binding protein may improve the efficiency of polymerisation. Subsequent addition of T4 or Tth, Pfu DNA ligase to the reaction joins the vector and insert improving transformation efficiency.

It will be evident to those skilled in the art that many alternative methods exist to create cDNA libraries such as those employing oligo dT, random primers, linkers, adaptors and RNaseH.

Appropriate *E.coli* hosts may include those that have reduced nuclease activity such as mutants for recB, recC, sbcB, nei, nfi, xth, nfo, hsd and/or those genotypes that increase the stability of clone inserts such as recA, recJ, sbcC, umuC and uvrC.

In a still further aspect, the present invention provides use of an oligo- or poly-nucleotide comprising RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position, in a hybridisation reaction.

In accordance with this aspect of the invention it has been surprisingly found that RNA modified in accordance with the present invention it is still capable of hybridising with nucleic acid. Because modified RNA is more stable to degradation than its unmodified counterpart, problems of degradation of RNA during and before analysis are avoided. There is no longer any need for extreme measures to be used to prevent RNA degradation such as those involving the use of ultra-clean working environments, or expensive inhibitors of RNases.

Typically, the hybridisation reaction comprises a hybridisation between a probe and a template comprising the oligo- or poly-nucleotide, which may comprise a mixture of

oligo- or poly-nucledotides such as those involved in a gene expression analysis.

Alternatively, the hybridisation reaction may comprise a hybridisation between a template and a probe comprising the oligo- or poly-nucleotide.

The probe or the template may be immobilised to a solid phase such as a hybridisation membrane, a bead, a particle, a sheet, a gel, a microtitre strip, tube, fibre or capillary.

The solid phase may be made of substances such as nitrocellulose, agarose, cellulose, latex, nylon, polystyrene, polycarbonate, PVDF (polyvinyldene fluoride), polytetrafluroethylene, a silica-based material, a metal allow, gold, a magnetic material or a paramagnetic material

The hybridisation reaction may comprise a blotting process typically using any one of the above solid phases.

The probe or template may be attached to another molecule or group of molecules. It is frequently desired that the probe or the template is labelled with a label, which may be a fluorescence label, a radioactive label, and enzyme, a ligand or an affinant for such a label. The molecules or group of molecules may itself comprise the label in the sense that the group of molecules is capable of causing a detectable reaction or capable of binding a detectable entity. The molecule or group of molecules may comprise a peptide, a poly-peptide such as an antibody, an enzyme, an affinity partner such as protein A or streptavidin, a

receptor protein, a ligand such as biotin, dinitrophenyl, digoxigenin or other hapten or lectin, or a label such as fluorescein, rhodamine, Texas red, cy-5, TAMRA or a pigmented chromogenic, chemiluminescent or coloured marker.

The probe may comprise a branched DNA (bDNA) probe.

In a further embodiment, the oligo- or poly-nucleotide may be bound to a third molecule such as an antibody-alkaline phosphatase conjugate.

The oligo- or poly-nucleotide may comprise an antisense agent for use in an antisense hybridisation reaction for example *in vivo*.

In accordance with a further use, the oligo- or poly-nucleotide has a specific binding affinity to a ligand and the hybridisation reaction comprises a hybridisation between the oligo- or poly-nucleotide and a target comprising the ligand.

Typically, the RNA comprises a ribozyme.

In a further aspect, the hybridisation reaction comprises a ligase chain reaction (LCR). LCR requires four specific oligonucleotides, DNA ligase and a DNA template. Typically, it relies upon the hybridisation of two template-specific oligonucleotides next to each other such that the 5'- phosphate of one adjoins the 3'-OH of the other. The two oligonucleotides are then ligated by a ligase and this ligated product serves itself as a template for further rounds of ligation in the presence of two

further oligonucleotides complementary to the first two oligonucleotides. Because initiation of LCR can only occur when a specific DNA template is present, LCR serves as an effective means for assay of such a template. According to the present invention, the template comprises RNA modified as described above.

In a further aspect, the hybridisation reaction comprises a nuclease protection assay in which unhybridised oligo- or poly-nucleotides are digested typically with a single stranded nuclease such as S1 nuclease or RNase T1, and the remaining oligo- or poly-nucleotide is analysed, usually by gel electrophoresis.

Nuclease protection assays thereby provide a means to quantitate mRNA abundance and to match the positions of exons, introns and 5' transcription start sites.

In a further aspect, the solid phase comprises a biochip. When the probe comprises the modified RNA typically labelled with a label, a starting mRNA population can be used to probe the biochip directly, following modification. Because there are no enzymatic steps required, the quantification of the mRNA transcript is improved. Alternatively, the target may comprise the modified RNA immobilised on discrete locations of the biochip or, alternatively, on discrete beads or particles. Because of a reduction in degradation of the RNA, gene expression analysis is improved.

In a further aspect, the probe is immobilised and comprises oligo- (dT), whereby the template is purified from

contaminants such as DNA. In this way, mRNA, for example, modified in accordance with the present invention can be sorted from the bulk of total RNA and/or DNA by means of its poly(A) tail. Hybridisation occurs between the modified poly(A) and the immobilised oligo(dT).

The modified RNA may also be used for diagnosis based on the presence or absence of a specified nucleotide sequence.

In a further aspect, there is provided a method for hybridising a first and second oligo- or poly-nucleotide which comprises contacting the first and second oligo- or poly-nucleotide under hybridisation conditions, wherein at least one of the first and second oligo- or poly-nucleotides comprises RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position.

This method advantageously further comprises obtaining at least one of the first and second oligo- or poly-nucleotides by (i) contacting in a reaction medium RNA comprising an oligo- or poly-ribonucleotide with a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA; (ii) reacting the RNA with the reactant to produce modified oligo- or poly-nucleotide under conditions to achieve covalent modification of greater than 75% of the 2'-OH positions of the ribose rings; and (iii) optionally separating the modified oligo- or polynucleotide from the reaction medium, wherein the reaction medium comprises an organic solvent. Preferably, the reaction medium comprise at least 20% v/v organic solvent, more preferably at least 50%, still more

preferably at least 80%, more preferably at least 90% and especially at least 95% organic solvent. The organic solvent advantageously comprises an organic base.

In a further aspect, the present invention provides a kit for modifying an oligo- or poly-nucleotide comprising an oligo- or poly-ribonucleotide, for use in a method as described above, which kit comprises

(a) an organic solvent; and

(b) a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the oligo- or poly-ribonucleotide in the presence of the organic solvent, wherein the reactant is labelled with a label.

The present invention may be used in a number of further applications including research applications and medical applications, as set out below.

Research Applications

Analysis of RNA structure and function

***In situ* Hybridisation**

The *in situ* hybridisation procedure relies on maintaining, in an intact form, cellular RNA especially mRNA in order to serve as a hybridisation partner for a RNA labelled probe. By examination of the localisation of the labelled probe, it is possible to identify specific tissues or cells where a particular gene is expressed. This procedure relies on both the target cellular RNA and the probe RNA being maintained in a largely intact form, otherwise hybridisation will not occur. Utility for the present invention may be found by stabilising both the target and

probe RNA such that they are not degraded as is common for unmodified RNA.

(1) The tissue sections that normally contain the target RNA could be treated prior to hybridisation by one or more reagents as have been described in examples 1-32. However, unlike examples 1-32, the target RNA would be treated in an unpurified form *in situ* with other cellular components such as the cell membranes, DNA and proteins. In this way, the entire RNA population is modified and therefore stabilised throughout the *in situ* hybridisation process.

(2) The normal form of the probe used for *in situ* hybridisation is a riboprobe produced by *in vitro* transcription and composed of a radioactively or fluorescently labelled single-stranded RNA. Such probes are liable to destruction at any point during the *in situ* procedure. Following the *in vitro* transcription reaction, the ribo-probe could be treated in a manner as described in one of the examples 1-32 in order to stabilise it against destruction. Such modified ribo-probes would retain their ability to interact in a specific manner with the target RNA. Alternatively, such modified ribo-probes could be used as probes for any number of hybridisation procedures such as northern and Southern blotting, chromosome mapping probes or any procedure which requires such probes.

RNA analysis Methods

Many techniques have been developed such as primer extension, S1 nuclease mapping and the RNase protection assay rely on intact RNA as a substrate for analysis. Degradation of the RNA will result in false quantitation of

RNA abundance or localisation of the structural features of the mRNA such as the 5' CAP site. Modification of the starting RNA to be analysed (primer extension) or the probe to be used for the analysis (S1 nuclease mapping) would lead to improved accuracy of the results.

RNA molecular weight markers

Molecular weight markers composed of RNA find use for calibrating northern blots and other procedures that separate RNA according to its size such as mass spectrometry. Commonly, RNA markers of discrete sizes are produced by an *in vitro* transcription reaction. However such RNA frequently becomes degraded during the separation procedure. In this invention, such discrete RNA molecules are treated in such a way as to maintain their intactness throughout the separation procedure.

Sequencing

There are two common methods for sequencing RNA, nuclease digestion and Maxam-Gilbert methods. The second method, employing reverse transcriptase would benefit from a modified RNA that is stabilised allowing greater quantities of cDNA and therefore sequencing product to be made.

Medical Applications

Modified RNA may interact with a target in two distinct ways. Firstly by hydrogen bonding (base-pairing) with a hybridisation partner (e.g. antisense oligonucleotides) or secondly by virtue of its secondary structure (e.g. aptamers). In either case, the modified RNA can find utility for therapeutics or diagnostics

Therapeutics

Any therapeutic molecule (such as antisense oligonucleotides) administered should have the following properties; (i) be resistant to *in vivo* degradation, (ii) be capable of crossing the cell membrane (i.e. show lipophillic properties), (iii) interact specifically and efficiently with the target molecule or cellular machinery, (iv) have a low toxicity and immunogenicity. By careful choice of the type of RNA modification it should be possible to meet many or all of these requirements. For example, a 2'-aliphatic chain would increase the lipophilic nature of the molecule whilst preventing degradation from RNases and retaining the ability to interact with a target.

Types of therapeutic molecules that could benefit in some way from the 2'-modification of RNA could include inhibitory molecules such as antisense oligonucleotides, aptamers and transcription factor decoys. Other types could be molecules with catalytic activity such as RNA enzymes (ribozymes) or RNA encoding specific peptides such as mRNA for use in gene therapy or nucleic acid vaccines.

Ribozymes

A catalytic RNA is called a ribozyme. It is capable of various reactions such as self cleavage or cleavage of a defined sequence in a heterogenous RNA. In this case, therapeutic activity could be associated with it if, it cleaved for example the HIV RNA genome. Other activities include binding to specific ligands with high affinity. An *in vitro* procedure has been designed to select RNA molecules with specific enzymatic functions. Modification of the 2'-OH group of such RNA molecules could endow it

with greater stability towards nucleases or indeed new enzymatic function.

Antisense

Antisense are sequences complementary to the sense strand of a mRNA and can consist of RNA, DNA or modified oligonucleotides. They interfere with the normal regulation and function of mRNA in such a way that the amount of protein synthesis is reduced. Through the interaction with the target RNA, protein translation is physically blocked or, RNaseH activity is triggered leading to the destruction of the target RNA. Such interference can have therapeutic effects if for example, viral mRNA sequences are targeted. Some of the theoretical advantages of such antisense therapy is their highly specific binding to target molecules and low toxicity.

Modified RNA antisense molecules might be expected to have enhaned activity compared with natural nucleic acids because they are more stable *in vivo* and/or are more liphophilic so that they enter the cell more readily.

Transcription Factor Decoys

Double stranded DNA representing a defined transcription factor binding site has been shown to be capable of binding nuclear proteins when delivered *in vivo*. Using this approach therapeutic results can be brought about by modifying gene activity via the removal of transcription factors from gene promoters. Considerable effort has been made in stabilising such double stranded DNA from the activity of cellular nucleases. Double stranded forms of modified RNA may be capable of binding transcription

factors *in vivo* and therefore serve as therapeutic agents because they will be refractive to the activity of most or all nucleases. Other advantages may include improved membrane permeability if lipophilic modifications are made to the RNA.

Enzyme Inhibitors and other Specific Binding Interations

The present invention further provides use of an oligo- or poly-nucleotide having a specific binding affinity to a ligand for binding specifically to a target comprising the ligand, wherein the oligo- or poly-nucleotide comprises RNA which is greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position.

RNA aptamers selected by the SELEX procedure could be used *in vivo* to inhibit the activity of key enzymes associated with a pathogenic organism such as reverse transcriptase or proteases of HIV. SELEX (Systematic Evolution of Ligands by Exponential Enrichment) can in theory, by starting with a sufficiently large pool of random RNA sequences, be used to select RNA molecules with any number of specificity's. For example RNA can be selected that is specific to the epidermal growth factor (EGF) or other hormones thus providing a potential therapeutic agent for blocking the activity of such hormones.

During the SELEX procedure, 2'-NH_2 groups 2'-F groups, 2'-methyl and 2'-*O*-methyl groups as ribonucleotides may be incorporated into the RNA chain by T7 RNA polymerase. See Gerard, et al., (1974) *Biochemistry* 13:1632; Jellinek, et al., (1995) *Biochemistry*. 34:11363; Pan, et al., (1995) *Proc. Natl. Acad. Sci. USA* 92:11509; Green, et al., (1995)

Chem. Biol. 2:683; Green, (1995) *J. Mol. Biol.* 247:60; Lin, et al., (1994) *Nucleic Acids Res.* 22:5229.

It has been shown that injection into animals of double stranded RNA which is complementary to cellular mRNA sequences can specifically interfere with the biological activity of the mRNA. The interference activity is far superior to either single stranded sense of anti-sense RNA. Such double RNA interference molecules have been called RNAi, Tabara et al., (1998) Science 282:430-431; Kennerdell and Cartew, (1998) Cell 95:1017-1026). As an alternative to double stranded RNA which might be expected to be rapidly degraded in a cellular environment, modified double stranded RNA could be used as RNAi molecules. These would be expected to have an equal biological activity as unmodified forms but be active for prolonged periods thereby improving their efficacy. Other than research applications, RNAi holds promise for therapeutic and other medical applications.

Diagnostics

Modified RNA can be used in a labelled form (e.g. radioactive or fluorescent labels) as a probe to monitor gene activity including the following applications, 'biochip' diagnostics, PCR, northern and Southern blotting, RNase protection, or any application where specific base-pairing is required between the probe and target.

In addition, labelled RNA with novel functions such as aptamers that can, like monoclonal antibodies bind to specific ligands, may be used as probes to localise a particular ligand within, for example a cell or tissue.

Medical imaging would be one such application for this technology. Suitably modified RNA that can bind a tumour marker for example would aid in the localisation of a cancer cells within the body or serve as an early indicator of cancer.

The invention will now be described in further detail, by way of example only, with reference to the following Examples and the accompanying drawings, in which:-

FIGURE 1a shows the ribose rings of RNA and DNA;
FIGURE 1b shows the mechanism of RNA strand cleavage;
FIGURE 2 shows RNA modified at the 2'-OH position;
FIGURE 3 shows masking and de-masking of RNA;
FIGURE 4 shows chemical modification and second strand polymerisation of RNA;
FIGURES 5a, b and c show respectively acylation, silylation and alkyl ether formation from RNA;
FIGURE 6 shows various modifications at the 2'-OH of RNA;
FIGURE 7 shows a trifluroacetic anhydride reaction of RNA;
FIGURE 8 shows formation of a 2'-chloro substituent on RNA;
FIGURE 9 shows a reaction of a phenoxy acetyl chloride reagent with RNA;
FIGURE 10 shows formation of a 2'-levulinate from RNA;
FIGURE 11 shows formation of a 2'-benzoyl from RNA;
FIGURE 12 shows a TBAF-catalysed silyl to hydroxyl replacement reaction;
FIGURE 13 shows a fluoride ion catalysed silyl to acyl replacement reaction;
FIGURE 14 shows allyl ether formation on RNA;
FIGURE 15 shows cleavage of a 2'-O-allyl ether group on RNA;

FIGURE 16 shows a sequencing gel demonstrating enhanced stability of modified RNA according to the invention;

FIGURE 17 shows a scheme of PCR and reverse transcription using modified RNA according to the invention;

FIGURE 18 shows a sequencing gel comparing PCR amplification products derived from modified RNA and DNA templates;

FIGURE 19 shows a comparison of hybridisation properties of modified and unmodified RNA;

FIGURE 20 shows results of agarose gel and northern blotting comparing modified and unmodified RNA;

FIGURE 21 compares gel electrophoretic migration behaviour of modified and unmodified RNA;

FIGURE 22 shows gel electrophoretic behaviour of butyric and pentanoic anhydride modified RNA;

FIGURE 23 shows gel electrophoretic behaviour of RNA acetylated in the presence and absence of a catalyst;

FIGURE 24 shows gel electrophoretic behaviour of RNA acetylated for different reaction times;

FIGURE 25 shows gel electrophoretic behaviour of RNA modified in the presence and absence of catalyst; and

FIGURE 26 shows electrophoretic behaviour of RNA treated in accordance with prior art and modified in accordance with the invention.

Example 1

Acetylation of total RNA followed by mRNA selection

The procedure for the modification of mRNA could be one of several. However a preferred method is as follows. The tissue such as 1g of mouse skeletal muscle is dissected and immediately snap-frozen in liquid nitrogen and then ground under liquid nitrogen with a mortar and pestle. Further

tissue and cellular disruption is then made by standard means such as homogenisation using a Waring blender (Waring Commercial of Gateshead, England), in the presence of guanidine isothiocyanate and phenol commercially available as TRIzol reagent (Gibco BRL). Alternatively, tissue culture cells from a 3.5 cm tissue culture plate can be homogenised in 1 ml of TRIzol reagent by passing them repetitively through a pipette. Following a 5 minute incubation at room temperature, 0.2 ml of chloroform was added per 1ml of TRIzol" reagent and shaken for 15 seconds. Following centrifugation at 12,000 x g for 15 minutes at 4°C the upper aqueous phase was removed and mixed with 0.5 ml of isopropanol per 1 ml of TRIzol" reagent in a fresh tube. The samples were incubated for 10 minutes at room temperature and centrifuged again at 12,000 x g for 10 min at 4°C. The pellet was washed with 1 ml of 75% ethanol per 1 ml TRIzol reagent, allowed to dry and redissolved in 0.2 ml water. The total RNA solution, comprised of 1-5 mg of tRNA, rRNA and mRNA fractions, is then added to 4ml TEA containing (12 mg; 98 µmoles) 4-dimethylaminopyridine (DMAP) and then 200 µl (1.96 mmoles) of acetic anhydride was added and mixed vigorously on ice for 1 minute then the reaction was allowed to proceed at room temperature for 2 minutes). The reaction was then terminated by addition of three reaction volumes of ethanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to ethanol precipitate the modified RNA by adding sodium chloride to a final concentration of 0.3 M and spinning in a centrifuge for 15 minutes at 14 000 x g at 4°C. (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH.) or Microcon-50 spin-column

purification (Amicon, USA) as described or another method that allows such purification. The modified RNA can then be used for any number of applications such as northern blotting or mRNA purification. The process of mRNA purification from the total acetylated fraction will now be described.

The acetylated mRNA fraction is separated from the acetylated total RNA by means of the poly(A) tail common to all mRNA molecules. Any number of methods have been described to purify mRNA, follows is the use of a commercially available kit for such a purpose. A PolyATract® isolation system from Promega, USA was used as follows. One milligram of total RNA is diluted into a final volume of 2.43 ml of water and incubated at 65°C for 10 minutes. Then 10 µl of biotinylated-oligo(dT) probe is added with 60 µl of 20 X SSC to the RNA solution and allowed to cool to room temperature over 30 minutes. The biotinylated-oligo(dT) probe - mRNA complex was mixed with 0.5 ml (0.5 X SSC) of streptavidin paramagnetic particles and incubated for 10 minutes at room temperature, then washed in 0.1 X SSC (4 X 1.5 ml). The mRNA fraction was then eluted by mixing the biotinylated-oligo(dT) probe-mRNA complex in 1 ml of water, removing the particles and collecting the aqueous phase. The acetylated mRNA thus prepared is suitable for applications including but not limited to cDNA library synthesis, northern blotting and in vitro protein translation. A yield of 30 µg mRNA from 1 mg of total RNA starting material is expected.

Example 2

Acetylation of purified mRNA

A sample of tissue such as 1g of mouse skeletal muscle is immediately snap-frozen in liquid nitrogen and then ground under liquid nitrogen with a mortar and pestle then transferred to a 10 ml centrifuge tube. Further tissue and cellular disruption is then made by standard means such as homogenisation using a Waring blender (Waring Commercial, Gateshead, England), in the presence of guanidine isothiocyanate and phenol commercially available as TRIzol reagent from Gibco BRL. Following a 5 minute incubation at room temperature, 0.2 ml of chloroform was added per 1ml of TRIzol reagent and shaken for 15 seconds. Following centrifugation at 12,000 x g for 15 minutes at 4°C the upper aqueous phase was removed and mixed with 0.5ml of isopropanol per 1 ml of TRIzol reagent in a fresh tube. The samples were incubated for 10 minutes at room temperature and centrifuged again at 12,000 x g for 10 minutes at 4°C. The pellet containing the total RNA fraction was washed with 1 ml of 75% ethanol per 1 ml TRIzol reagent, allowed to dry and redissolved in 0.2 ml water.

The mRNA fraction is separated from non-polyadenylated RNA by any number of methods such as the PolyATract® isolation system from Promega, USA which was used as follows. One milligram of total RNA is diluted into a final volume of 2.43 ml of water and incubated at 65°C for 10 minutes. Then 10 µl of biotinylated-oligo(dT) probe is added with 60 µl of 20 X SSC to the RNA solution and allowed to cool to room temperature over 30 minutes. The biotinylated-oligo(dT) probe - mRNA complex was mixed with 0.5 ml (0.5 X

SSC) of streptavidin paramagnetic particles and incubated for 10 minutes at room temperature, then washed in 0.1 X SSC (4 X 1.5 ml). The mRNA fraction was then eluted by mixing the biotinylated-oligo(dT) probe-mRNA complex in 0.2 ml of water, removing the particles with the magnetic stand and collecting the aqueous phase.

To 0.1-1µg of mRNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (9.8 µmol) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g. Recovery volumes were typically 5-15 µl and recovery yields >95%.

Example 3

Halide ion-catalysed acetylation reaction

The specificity and amount of mRNA acetylation can be improved by the addition of halide ions such as fluoride ions. Between 100 ng to 1000 ng of purified mRNA was mixed with a solution containing 30 nmol tetrabutylammonium fluoride (TBAF) or tetrabutylammonium iodide (TBAI), 10 µmol of acetic anhydride and tetrahydrofuran (THF) or triethylamine (TEA) serving as the solvent to bring the final volume to 20 µl. The reaction is allowed to proceed for 2 to 30 minutes at room temperature. Alternatively, pivalic anhydride or benzoic anhydride may be substituted for the acetic anhydride as the acetyl donor (Beaucage and Ogilvie, (1977) Tetrahedron Lett., 1691). Alternatively 10 µl of one of the acylating reagents, propanoic, butyric, pentanoic, hexanoic, heptanoic, octanoic or benzoic anhydrides was added instead of acetic anhydride. All other reaction and purification methods were identical. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 4

Aminopyridine-catalysed acylation reaction

Described is the catalytic acylation of alcohols with an acid anhydride involving triethylamine and the hypernucleophillic acylation catalyst aminopyridine such as 4-pyrrolidinopyridine. To a solution of 1 µg of RNA in 1 µl of water was added 60 µg of 4-pyrrolidinopyridine in 20 µl triethylamine(TEA) and then 10 µmol of an acid anhydride

such as acetic, propanoic, butyric, pentanoic, hexanoic, heptanoic, octanoic or benzoic anhydrides was added. The reaction was mixed and allowed to proceed at room temperature until acetylation was complete (2 minutes to 30 min), (Hofle and Steglich, (1972) Synthesis 619; Steglich and Hofle, (1969) Tetrahedron Lett. 4727; Hassner, et al., (1978) Tetrahedron 34:2069). Excess components of the reaction were removed either by ethanol precipitation (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 5

RNA labelling with a fluorescent or radioactive group

The modifying chemical used to react with the 2'-OH group could include a radioactive label such as 14C, tritium, (3H) or a fluorescent marker such as fluoroscein or rhodamine, as a means to label the molecule at multiple positions. Suitable labelled reactants include 14C- or 3H-acetic anhydride and are used a follows. To 1 µg of mRNA was added 20 µl of triethylamine containing (60 µg; 490 nmol) DMAP and 500µCi of 14C (100-124 µCi/mmol) acetic anhydride (Amersham, UK).

The unreacted components including the radiolabelled acetic anhydride were removed either by ethanol precipitation (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The specific activity of the labelled RNA is quantified by TCA precipitation. The purified radiolabelled mRNA is suitable for a variety of purposes as a hybridisation probe.

Example 6

DMAP-catalysed acylation reactions

To 0.1-1µg of mRNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g. Recovery volumes were typically 5-15 µl and recovery yields >95%.

Alternative acetylating reagents were used with the same protocol except a maximum of 200 ng of RNA was used per reaction. In each case 10 µmol of the acylating reagents from the list, propanoic, butyric, pentanoic, hexanoic, heptanoic, octanoic or benzoic anhydrides was added instead of acetic anhydride. All other reaction and purification methods were identical.

Example 7

Trifluoroacetic anhydride reactions

To 0.1-1µg of mRNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (4.8 µmol) of trifluoroacetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g.

Example 8

DMAP-catalysed acid chloride reactions

To 0.1-1.0µg of mRNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (3.5 µmol) of a solution of 25% acetyl chloride in toluene was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction

was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g. Recovery volumes were typically 5-15 µl and recovery yields >95%.

Example 9

Condensation reactions between carboxylic acid and RNA

In order to promote the esterification process, dehydrating agents such as *N,N'*-dicyclohexylcarbodiimide (DCC) are used. 1µg of mRNA (6pmol) was dissolved in 10nmol at carboxylic acid containing 11 nmol dicyclohexylcarbodiimide (DCC) 1 nmol of 4-pyrrolidinopyridine and ether or dichloromethane was added to bring the final volume to 50 µl. The reaction was allowed to proceed at room temperature until esterifiction was complete (20 min - 6 hrs). The carboxylic acids used can be benzoic, acetic, diphenylacetic and mesitoic (Hassner and Alexanian, (1978) *Tetrahedron Letters* 4475). The nucleic acid fraction of the reaction was purified either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described In order to improve the solubility of the RNA it can be dissolved in 10 µl of

either dimethyl formamide or dimethyl sulphoxide before adding it to the reaction.

Example 10

t-Butyl isocyanide-catalysed acylation with carboxylic acid

The use of isonitrile reagents such as t-butyl isocyanide in the esterification of alcohols with carboxylic acids (Rehn and Ugi, (1977) *J. Chem Research* (M) 1501-1506). 3.5µg mRNA (6 pmol) was dissolved in a solution containing 5 nmol carboxylic acid, 15 nmol t-butyl isocyanide and either ether or dichloromethane to bring the final volume to 50 µl. The reaction was allowed to proceed at room temperature until esterifiction was complete (approximately 3 hrs). The carboxylic acids used can be, but are not restricted to acetic, diphenylacetic and mesitoic. The nucleic acid fraction of the reaction was purified either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. In order to improve the solubility of the RNA it can be dissolved in 10 µl of either dimethyl formamide or dimethyl sulphoxide before adding it to the reaction.

Example 11

Use of phenylphosphine

Triphenylphosphine can be used for transformation of alcohols into alkyl chlorides with carbon tetrachloride (Regen, (1975) *J. Org. Chem.* 40:1669. To 3.5 µg mRNA (6 pmol) was added 1.25 µg (4 nmol of phosphorus) of polystyryl-diphenylphosphine in 50 µl of carbon tetrachloride. Following mixing, the reaction was allowed to proceed at room temperature until alkyl chloride

formation was complete (20 min - 6 hrs). The reaction mixture was filtered to remove the polystyryl-diphenylphosphine using an appropriate spin-column filter such as an Amicon 0.22 µm Micropure™ according to the manufacturer's instructions. The nucleic acid fraction of the reaction was then purified either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 8. In order to improve the solubility of the RNA it can be dissolved in 10 µl of either dimethyl formamide or dimethyl sulphoxide before adding it to the reaction.

Example 12

Use of phenoxyacetyl chloride reagents

Several carboxylic acid reagents have been developed for application in nucleoside/nucleotide synthesis including various phenoxyacetyl chlorides (Reese, (1978) *Tetrahedron* 34:3143 ; Jones and Reese, (1979) *J. Amer. Chem. Soc.* 101:7399). The RNA (1 µg;6 pmol) in 10 µl dimethyl formamide was added to 50 µl of pyridine containing 2-chlorophenyl 4-nitrophenyl phosphorochloridate. The reaction was allowed to proceed at room temperature until phosphotriester formation was complete (approximately 2 hrs). The nucleic acid fraction of the reaction was then purified either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 9. In order to improve the solubility of the RNA it can be

dissolved in 10 µl of either dimethyl formamide or dimethyl sulphoxide before adding it to the reaction.

Example 13

Use of levulinic acid reagents

1 µg (1.7 pmol) of RNA was dissolved in 10 µl of dimethyl formamide and then dioxan containing 3.4 nmol of levulinic acid, 3.4 nmol of DCCI and 100 µg of DMAP was added and mixed. The reaction was allowed to proceed for 24 hr at room temperature. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 10.

The levulinate group can subsequently be removed by two alternative methods. Method (1). By the addition of 47.7 µg of sodium borohydride ($NaBH_4$) to a 50 µl solution containing 10 µl water and 40 µl of dioxan and the levulinate RNA. The pH is brought to 5 by the addition of acetic acid and the reaction allowed to proceed at room temperature for 6 hr. Method (2). 1 µg of the levulinate RNA was treated with 10 µl of 10 mM hydrazine hydrate in pyridine - acetic acid (4:1 vol/vol)(van Boom and Burgers, *Tetrahedron Letters* (1976) 4875). In both cases the mRNA was recovered by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH). In order to improve the solubility of the RNA it can be dissolved in 10 µl of either dimethyl formamide or dimethyl sulphoxide before adding it to the reaction.

Example 14

Use of benzoic acid reagents

The tetrazole system activates the benzoyl group to create a simple benzoyl-transfer reagent, and benzoic acid reagents, such as 1-benzoyltetrazole, (Stawinski, et al., (1976) *J. Chem. Soc. Chem. Commun.* 243). To a solution containing 3.5 µg mRNA (6 pmol) dissolved in 40 µl dimethyl formamide was added 4 nmol of 1-benzoyltetrazole and a catalytic amount (0.6 pmol) of triethylamine. The reaction was allowed to proceed at room temperature until complete (approximately 30 min). The nucleic acid fraction of the reaction was then purified either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 11.

Example 15

2'-*O*-Silylation approaches

To a solution containing 1 µg mRNA (6 pmol) was added 7 nmol of the silyl chloride reagent (either methyldiisopropylsilyl (MDIPSI), 1-(Trimethylsilyl)imidazole (TMSIM), TMIPSi, *t*-butyldimethylsilyl (TBDMS), TIPSi or TMTBS), 14 nmol of imidazole (Murata and Noyori, (1980) *Tetrahedron* 21:767; Ogilvie, et al., (1974) *Tetrahedron Letters* 2865; Usman et al., (1987) *J. Am. Chem. Soc.* 109:7845-7854). The reaction was allowed to proceed at room temperature until esterifiction was complete (20 min-6 hrs). The nucleic acid fraction of the reaction was purified by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*,CSH). In order to improve the solubility

of the RNA it can be dissolved in 10 µl of either dimethyl formamide or dimethyl sulphoxide before adding it to the reaction.

Example 16

DMAP-catalysed 2'-*O*-Silylation

To a solution containing 50 µl of triethylamine containing 1µg of DMAP was added 1 µg of RNA and 200 nmol of the silyl chloride (either methyldiisopropylsilyl (MDIPSI), TMIPSi, t-butyldimethylsilyl (TBDMS), TIPSi and TMTBS), (Shiao, et al., (1988) *Synthetic Comm.*. 18:359). The reaction was allowed to proceed at room temperature for 16 hrs. The nucleic acid fraction of the reaction was purified by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH).

Example 17

TBDMS triflate 2'-*O*-Silylation

1 µg mRNA (1.6 pmol) was dissolved in 10 µl of dimethyl formamide and then added to 40 µl of dichloromethane at 0°C under nitrogen and then 6 nmol of 2,6-lutidine and 3 nmol of t-butyldimethylsilyl trifluoromethane triflate (TBDMS triflate). The reaction was proceeded at 0°C for 1 hr. The nucleic acid fraction of the reaction was purified by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH).

Example 18

Lithium sulphide-catalysed 2'-*O*-Silylation

1 µg mRNA (1.6 pmol) was dissolved in 10 µl of dimethyl formamide and then added to 40 µl of acetonitrile

containing 3-5 µg mRNA (6 pmol) chlorotrimethylsilane (10 nmol) or t-butyldimethylsilyl chloride (10 nmol), lithium sulphide (4 nmol) and acetonitrile to bring the final volume to 50 µl. The reaction was allowed to proceed at room temperature until complete (Olah, et al., (1979) *J. Org. Chem.* 44:4272). The nucleic acid fraction of the reaction was purified either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 19

TBAF-catalysed silyl to hydroxyl replacement

The reaction leads to the direct replacement of a 2'-*O*-silyl group with the original hydroxyl group when carried out in the presence of fluoride ion. Between 100 ng to 1000 ng of silyated mRNA in 10 µl of dimethyl formamide was mixed with a solution containing 150 nmol tetra-(n-butyl) ammonium fluoride (TBAF) and tetrahydrofuran (THF) serving as the solvent to bring the final volume to 50 µl. The reaction is allowed to proceed for 24 hours at room temperature. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 12.

Example 20

TEA.3HF-catalysed silyl to hydroxyl replacement

The reaction leads to the direct replacement of a 2'-*O*-silyl group with a hydroxyl group when carried out in the presence of TEA.3HF. Between 100 ng to 1000 ng of silyated

(TBDMS) mRNA in 10 µl of dimethyl formamide was mixed with 50 µl of pure triethylamine-tris-hydrofluoride (TEA.3HF). The reaction is allowed to proceed for 14 hours at room temperature (Sproat, et al., (1995) *Nucleosides and Nucleotides* 14:255). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 21

Fluoride ion-catalysed silyl to acyl replacement

The reaction leads to the direct replacement of a 2'-*O*-silyl group with an 2'-*O*-acyl group when carried out in the presence of fluoride ion. Between 100 ng to 1000 ng of silyated mRNA in 10 µl of dimethyl formamide was mixed with a solution containing 30 nmol tetra-(n-butyl)ammonium fluoride (TBAF), 75 nmol of acetic anhydride and tetrahydrofuran (THF) serving as the solvent to bring the final volume to 50 µl. The reaction is allowed to proceed for 30 minutes to 5 hours at room temperature. Alternatively, pivalic anhydride or benzoic anhydride may be substituted for the acetic anhydride as the acetyl donor or acetylating agent (Beaucage and Ogilvie, (1977) *Tetrahedron Letters*, 1691). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 13.

Example 22

Phase transfer catalysis-2'-*O*-Alkylation approaches

To the two phase system consisting of 1 µg (1.6 pmol) of mRNA was added 6 ng of tetrabutylammonium iodide in 5 µl of dichloromethane and 2.5 µl of 7.8 nmol NaOH was vigorously mixed for 30 minutes and then 4 nmoles of either dimethyl or diethyl sulphate was added whilst the reaction temperature was maintained at 45°C. The reaction was allowed to proceed for 3 hours at 45°C and then 1 µl of NH_3 added, stirred and incubated for 30 minutes at room temperature (Merz, (1973) . *Angew. Chem. Intl. Edit.* 12:846). The unreacted components were removed from the methyl ether modified RNA by either ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. Alternatively, 6 ng of another phase transfer catalyst such as tetrabutylammonium bromide, tetrabutylammonium hydrogensulphate or tetraethylammonium tetrafluoroborate could be used.

Example 23

Dialkyl sulphate reactions

To 1 µg (1.6 pmol) of mRNA was added 20 µl of dimethylformamide, and 4 nmoles of either dimethyl or diethyl sulphate was added whilst the reaction temperature was maintained at room-temperature. The reaction was allowed to proceed for 3 hours at room-temperature (Tazawa, et al., (1972) *Biochemistry* 11:4931). The unreacted components were removed from the methyl ether modified RNA by either ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH)

or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 24

Diazomethane for 2'-*O*-methyl formation

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 7 nmol of a diazomethane in a total volume of 50 µl of 1,2-dimethoxyethane. All reaction components were mixed on ice and the reaction was allowed to proceed for 24 hr at room temperature. (Broom and Robbins, (1965) *J. Am. Chem. Soc.* 87:1145-1146; Khwaja, et al., (1966) *J. Am. Chem. Soc.* 88:3640-3643; Martin, et al., (1968) *Biochem.* 7:1406-1412; Gin, (1968) *Biochem.* 7: 1413-1420; Robins, et al., (1971) *Biochem.* 10:3591-3597). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 25

Diazomethane and $SnCl_2$ for 2'-methyl ether formation

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 7 nmol of a diazomethane, 1 ng of $SnCl_2$ in a total volume of 50 µl of 1,2-dimethoxyethane. All reaction components were mixed on ice and the reaction was allowed to proceed for 24 hr at room temperature (Robins, (1974) *J. Org. Chem.* 39:1891-1899; Ekborg, (1980) *J. Carbohydrates Nucleosides Nucleotides* 7:57-61; Robins, (1981) *Can. J. Chem.* 59:3360-3364). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory*

Manual, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 26

Use of Methyl Iodide for 2'-methyl ether formation

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 7 nmol of a alkyl iodide such as methyl iodide, 1 ng of Ag_2O in a total volume of 50 µl of dimethylformamide. All reaction components (Purdies method) were mixed on ice and the reaction was allowed to proceed for 24 hr at room temperature in the dark (Furukawa, Y. et al. (1965) Chem. Pharm. Bull. 13:1273; Frukawa, (1965) *Chem. Pharm. Bull.* 13:1273-1278; Inoue, (1987) *Nucleic. Acid. Res.* 15:6131-6148). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 27

Alkyl Halides

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 12 nmol of phosphorus tribromide or phosphorus trichloride in a total volume of 50 µl of ether. All reaction components were mixed at room temperature and the reaction was allowed to proceed for 4 hr at 35°C. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 8.

Example 28

Thionyl chloride-Alkyl Halides

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 6 nmol of thionyl chloride ($SOCl_2$) in a total volume of 50 µl of pyridine. All reaction components were mixed at room temperature and the reaction was allowed to proceed for 4 hr at 35°C. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 29

2'-*O*-Alkylation approaches - Allyl ether formation

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide in 10 µl of dry benzene was added 0.2 µl of allyl bromide and 1 µg of NaOH. All reaction components were mixed on ice and the reaction was then allowed to proceed for 4 hours at 60°C (Gigg and Warren, (1969) *J. Chem. Soc.* C 2367-2371). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described. The reaction is shown schematically in Figure 14.

Example 30

Cleavage of a 2'-*O*-allyl ether group

Between 100 ng to 1000 ng of the allyl ether-modified mRNA in 10 µl of dimethyl formamide was mixed with a solution containing 0.2 nmol tristriphenylphosphine rhodium chloride ($RhCl(PPh_3)_3$), 0.6 nmol of diazabicylo[2.2.2]octane in 10%

ethanol and heated at 60°C for 3 hr (Corey and Suggs, (1973) *J. Org. Chem.* 38:3224). The pH of the reaction is then adjusted to 2 using small aliquots of 1N HCl and after 3 min the reaction was neutralised using Tris HCl-buffered water (pH 8). The mRNA was separated by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH). The reaction is shown schematically in Figure 15.

Example 31

Lipophillic Ester

To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 0.2 µl of sodium hydride and 1 µg of hexyl bromide. All reaction components were mixed on ice and the reaction was then allowed to proceed for 4 hours at room-temperature (Manoharan, *Biorganic and Medicinal Chem. Letters.* (1993) 3:2765). The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

Example 32

Northern blotting

A sample of the modified mRNA was prepared as in example II, 1µg was loaded on a denaturing agarose gel, with electrophoresis and transfer to a membrane as described (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH). The membrane was then used in a hybridisation with a radioactively labelled probe using standard methods. Alternatively, following transfer and immobilisation of the modified mRNA to the membrane the

acetyl or other ammonia sensitive group at the 2' position was cleaved with 28% ammonium hydroxide as follows. The membrane was covered with 50 ml of concentrated ammonia and incubated at room temperature for 5 minutes. In this case, the acetyl group at the 2'-position (i.e. 2'-O-COR) is replaced by the original 2'-OH group and therefore has normal hybridisation properties.

Example 33

Acetylation using Imidazole reagents

To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (150 µg) of *N*-acetylimidazole (Review: Newer Methods of Prep. Org. Chem. 5:61) was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was purification using a Centricon-50 spin-column (Amicon, USA). Alternative imidazole reagents such as N-benzoylimidazole could be used used with the same protocol.

Example 34

Fluorescent Labelling of RNA

Derivatives of isatoic and *N*-methylisatoic anhydrides are fluorescent (Hiratsuka (1982) J. Biol. Chem. 257:13354). Fluorescent RNA derivatives are useful as probes for hybridisation studies such as Southern blotting and other

applications. To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (10 µmoles) of either isatoic anhydride or *N*-methylisatoic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 minutes at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Fluorescent RNA was removed from the reactants and solvent using one of several methods. The preferred method was purification using a Centricon-50 spin-column (Amicon, USA). The exitation wavelengths were 330 nm for isatoic anhydride derivatives and 350 nm for *N*-methylisatoic anhydride derivatives with emision spectra in the range of 410-445 nm depending on the solvent polarity.

Example 35

Acetylation using Tributylphosphine catalyst

To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of tributylphosphine (Vedejs and Diver (1993) J. Am. Chem. Soc. 115:3358) and then 1 µl (10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several

methods. The preferred method was purification using a Centricon-50 spin-column (Amicon, USA).

Example 36

Uncatalysed Acetylation

To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing 1 µl (10 µmoles) of acetic anhydride. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was purification using a Centricon-50 spin-column (Amicon, USA).

Example 37

Overnight reaction using reduced acetic anhydride amounts

To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 0.1 µl (1 µmol) or 0.01 µl (0.1µmol) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for overnight at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds.

Example 38

Mixtures of acetylating reagents

To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl of a mixture of 9 parts acetic anhydride (8.82 µmole) and 1 part acetyl chloride (1.4 µmole) was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for over-night at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds.

Example 39

Mixtures of modifying reagents

In certain circumnstances where it is desirable to obtain RNA modified with two or more modifying groups, mixtures of modifying reagents can be used in the same reaction. The relative proportion of reactivity of each reagent will determine the final number of each modifying group attached to each RNA chain. To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (5 µmole) of a mixture of 1 part acetic anhydride and 1 part (5 µmole) propionic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed over-night at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. In principle any combination of reactants could

be mixed, providing there is no interaction between them, to give a wide range of multiply modified RNA.

Example 40

Diluting Acetyl chloride

It was found that adding 1 µl of acetyl chloride undiluted directly into the reaction led to excessive production of a white precipitate which made handling of the liquid difficult. For this reason acetyl chloride was first diluted in a suitable solvent such as toluene before mixing with the RNA. To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl of either 10% (1.4 µmole) or 25% (3.5 µmole) acetyl chloride diluted in toluene was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for over-night at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds.

Example 41

Protection with Crown 18-6

It has been reported that addition of crown 18-6 eliminates the reaction of acetic anhydride with primary amines (Barrett et al., (1978) J. Chem. Soc. Chem. Commun. 471). In order to test the effect of crown 18-6 addition on RNA acetylation, it was added in varying amounts to a standard acetylation reaction. To 0.1-1µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine

(DMAP), and either 6.6 µg, 660 ng, 66 ng, 6.6 ng, 660 pg or 66 pg of crown 18-6 added and allowed to complex for 5 min. at room temperature and then 1 µl (10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 20 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds.

Example 42

Increased RNA Substrate Quantity

To determine the upper limit for the amount of RNA that can be added to a standard acetylation reaction, varying amounts of RNA were added. The highest RNA concentration used (24 µg) represented the highest concentration of RNA that it was possible to dissolve in 1 µl of water without the RNA precipitating out of solution. To either 0.5, 1, 2, 6, 12 or 24 µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 20 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds.

Example 43

Deprotection with Alkali

The reversible nature of the acetylation reaction was examined using alkali which is known to lead to the cleavage of the acetyl group and replacement with a -OH group. To 0.1-1µg of acetylated RNA in 5 µl of water was added 1 µl of either 1 M, 500 mM or 250 mM of freshly dissolved NaOH and deprotection allowed to proceed for 15 min. at room temperature before neutralisation with an equal volume and concentration of HCl. It was found that subsequent to acetyl group cleavage, the 2'-OH group was attacked by the alkali leading to the RNA phosphate backbone cleavage.

Example 44

Deprotection with Ammonium Hydroxide

Ammonium hydroxide is also an effective reagent for acetyl group cleavage. To 0.1-1µg of acetylated RNA in 5 µl of water was added 1 µl or 5 µl of ammonium hydroxide solution (26%) and deprotection allowed to proceed for 15 min. at room temperature. The RNA can be purified using a Centricon-50 column. Other acyl groups such as those produced by reaction of butyric or propionic anhydrides will also be cleaved by this ammonia hydroxide treatment.

Example 45

Modified RNA Stability

The instability of RNA is a consequence of the reactivity of the ribose 2'-OH groups which leads to strand breakage. Many conditions and chemicals lead to the RNA strand breakage such as high pH and divalent metal ions. The consequence of modifying the 2'-OH group is therefore to

increase the stability and intactness of the RNA allowing complete cDNA copies and accurate measurements of its size and abundance to be made.

Experimental Approach

The key physical characteristic of RNA is its intactness and completeness. Natural RNA chains can be tens of thousands of bases long and must be preserved in this condition if they are to be usefully studied. In order to measure the robustness of modified RNA under conditions known to lead to RNA chain cleavage a sequencing gel assay was used. Simply, a radioactive nucleotide was incorporated into RNA during an *in vitro* transcription reaction, then the RNA modified and subjected to conditions that normally results in its degradation. The result was analysed following electrophoresis on a sequencing gel to assess its completeness. Intact (modified) RNA gave a single band whilst degraded RNA appeared as a smear of smaller fragments. In each case, RNA modified with various reagents were compared side by side with natural RNA. An added advantage of this gel assay was the unambiguous identification of modified RNA sample lanes because of its reduced electrophoretic mobility (see figure below).

Figure 16 demonstrates the enhanced resistance of modified RNA. A sequencing gel was run with alternating lanes of normal RNA (lanes 1,3,5,7) and acetylated RNA (lanes 2,4,6,8). Two RNA sizes of 250 and 1525 bases can be seen per lane. Samples were heated in a PCR buffer (2.5 mM MgCl2) for 0 min (lane 1,2), 2 min. (lane 3,4), 6 min. (lane 5,6), and 13 min. (lane 7,8), at 94°C. Despite less modified RNA than normal RNA was loaded (compare lanes

1 and 2), acetylated RNA was still detectable after 13 minutes at 94°C whilst normal RNA was undetectable after only 6 minutes. Note the smear in lane 3 as a result of RNA degradation.

Enzymatic Degradation of RNA Samples

A selection of commonly used nucleases were incubated with labelled RNA samples and the degradative effect visualised by sequencing gel degradation. The enzymes and conditions used were; S1 nuclease (Part. No. E576A, Promega, USA), degrades single-stranded DNA and RNA. 100 ng of each type of RNA was mixed with 10 µl of 1 x S1 nuclease buffer containing 15, 1.5 or 0.15 units S1 and incubated for 15 min at 37°C. Mung bean nuclease (part. No. M194A, Promega, USA) an endonuclease, degrades single stranded DNA and RNA. 100 ng of each type of RNA was mixed with 10 µl of 1 x mung bean nuclease buffer containing 50, 5 or 0.5 units nuclease and incubated for 15 min at 37°C. 10-100 pg RNase A (Cat. No. 109 142, Boehringer Mannheim) a general purpose RNase was incubated with 100 ng each RNA in 1 x buffer (40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine and 10 mM NaCl) and incubated for 15 min at 37°C. 1 / 1000 unit RNase One™ (Part. No. M4261, Promega, USA) was mixed with 100 ng of each RNA in 1 x RNase One buffer and incubated for 15 min at 37°C.

Chemical Degradation of RNA Samples

Conditions known to favour RNA degradation include high pH and metal ions. In each case 100 ng of each labelled RNA sample (modified and normal) was mixed under the following conditions. An equal volume of formamide and RNA sample was mixed and incubated for 1 min to 30 min at 99°C. A 9

mM $MnCl_2$ solution was mixed with each RNA to bring the final Mn^{2+} concentration to 1.5 mM. The mixture was then heated for 5 min at 100°C. A 25 mM $MgCl_2$ solution was mixed with each RNA to bring the final Mg^{2+} concentration to 1.5 mM. The mixture was then heated for 5 min at 100°C. 100 ng of each RNA was incubated with 100 mM, 250 mM, 500 mM and 1 M NaOH solution and incubated for 5 min at room temperature. 100 ng of RNA was mixed with 10 µl of PCR buffer (15 mM Tris-HCl pH 8.8, 60 mM KCl, 2.5 mM $MgCl_2$) and heated for 5 min at 94°C followed by 15 min at room temperature. Serum assays were carried out by removing the red blood cell component from 200 µl human blood and incubating 100 ng RNA with 10 µl serum for 15 min at 37°C.

Table 1. Comparison of susceptibility of modified and normal RNA to degradation.

Reagent ↓	NaOH	Mn2+	Mg2+	Formamide	RNase A	S1	MBN	RNase One	PCR Rxn	Serum
Normal RNA	x	x	x	x	x	x	x	x	x	x
Acetic anhydride	√	√	√	√	x	x	±	√	√	x
Propionic anhydride	√	-	-	-	-	x	±	√	√	-
Butyric anhydride	√	-	-	-	-	√	±	√	√	-
Pentanoic anhydride	√	-	-	-	-	√	±	-	-	-
Hexanoic anhydride	√	-	-	-	-	-	-	-	-	-
Heptanoic anhydride	√	-	-	-	-	-	-	-	-	-
Octanoic anhydride	√	-	-	-	-	-	-	-	-	-
Benzoic anhydride	√	-	-	-	-	-	-	-	-	√
Trimethyl acetic anhydride	√	-	-	-	-	-	-	-	-	√

Abbreviations for Table 1. NaOH 100 mM, Mn^{2+} 1.5 mM, Mg^{2+} 1.5 mM, Formamide 99°C, RNase A 100 pg, S1 Nuclease 15 units, MBN (mung bean nuclease) 50 units, RNase One 1 / 1000 unit, PCR reaction 94°C and serum 15 min at 37°C. Symbols: **X** RNA degraded, √ RNA shows good resistance to degradation, ± moderate resistance, - not assayed.

Conclusion

The modification of the ribose 2'-OH group provides excellent resistance to conditions that would otherwise lead to the rapid degradation of RNA. Carbon chain lengths that were attached to the 2'-OH group of the test RNA were from 2 carbon (acetyl) to 8 carbon (octoanate) and trimethyl acetyl and the benzoyl group. The 8 carbon chain length was not preferred because the extent of RNA modification was below 100%. Carbon chain lengths of 3-5 were preferred because they efficiently modified the RNA and offered good protection from both enzymatic and chemical attack. Protection from degradation in 1 x PCR buffer is significant because PCR amplification involves heating samples in Mg buffers; conditions that rapidly lead to degradation of RNA. Protection offers the opportunity to use modified RNA directly as a template for PCR amplification.

Example 46

PCR and Reverse Transcription

EXPERIMENTAL

Template Preparation

Synthetic RNA transcripts were used as templates because they have completely defined sequences with known annealing sites for DNA primers. RNA templates derived from an *in vitro* transcription reaction using T7 RNA polymerase and pGEM express positive control template (Part No. P256A, Promega, USA) were prepared according to the manufacturer's instructions. Two RNA transcripts were generated of 1065 and 2346 bases in length (see Figure 17). Template DNA was removed by the addition of 1 unit of RNase free DNase RQ1 and incubating for 15 min at 37°C, followed by extraction

with phenol:chloroform, then chloroform:isoamyl alcohol (24:1) and a final purification using Centricon-50 column filtration (Amicon, USA). Final volumes were typically 10 µl and RNA concentrations adjusted to 1 µg/µl. This procedure provided very pure RNA preparations suitable for chemical modification and subsequent use as DNA polymerase templates.

The use of DNA primers specific for the RNA modification that had been prepared resulted in newly synthesised DNA strands of pre-determined sizes thereby aiding analysis. The 1065 and 2346 base RNA transcripts prepared as described above contain annealing sites for the primers SP6 and T3. SP6 and T3 could be used together for PCR or SP6 alone for reverse transcription studies.

PCR Amplification

The PCR was carried out in a final volume of 25 µl with final concentration of 15 mM Tris-HCl pH 8.8, 60 mM KCl, 2.5 mM $MgCl_2$, 400 µM each dNTP, 10 pmol of each primer SP6 and T3 and 1 unit *Taq* DNA polymerase (Amersham, UK). Generally 10 ng of template DNA, RNA or modified RNA was added per reaction. Cycle parameters were 94°C x 20 sec, 55°C x 20 sec and 72°C x 30 sec for 30 cycles. PCR products were visualised following gel electrophoresis and staining with EtBr.

Reverse Transcription

100 ng (modified or normal) RNA was heated for 10 min at 75°C in 10 µl of water containing 50 ng SP6 primer or oligo (dT) and then left on ice. To this was added 2 µl of 25 mM $MgCl_2$, 2 µl 100 mM DTT, 1 µl 10 mM dNTPs, 1 µl 32P dCTP and

1 µl (10 units), Superscript II (Gibco-BRL, USA) (10 units), HIV reverse transcriptase (Seikagaku, Japan) or (10 units) AMV reverse transcriptase (Invitrogen, Netherlands). The reaction was incubated at a temperature from 37°C to 55°C for 30 minutes and stopped by the addition of 1 µl of 0.5 M EDTA. TCA precipitation was carried out by spotting 5 µl of the reaction onto glass filters and washing three times with 100 ml 10% TCA and counting. For gel analysis, the reaction was mixed with one volume 95% formamide load dye containing bromophenol blue and loaded into a 7M urea, 4% acrylamide gel containing 1 x TBE and run at 80 W for 1 hour. The gel was then fixed for 5 minutes in 10% acetic acid and dried. Bands were quantitated using a Molecular Dynamics Phosphorimager.

Sequencing

PCR products derived from modified RNA templates were purified by electrophoresis on a 1.2% agarose gel in 1 X TAE buffer and Geneclean according to the instructions of the manufacturer (Bio 101 Inc, La Jolla, CA, USA). The PCR product was eluted into 17 µl of H_2O at 55°C for 5 min and 7 µl were sequenced using the standard Sequenase protocol (U.S. Biochemicals, Cleveland, OH, USA). The annealing reaction was prepared by combining 7 µl of purified PCR product (0.5-5 µg) and 200 ng of T3 or T7 primer in a total volume of 8 µl of 10% DMSO. The DNA was denatured by incubation at 94°C for 1 min and snap frozen on dry ice. After removal from the dry ice the annealing mix was thawed by the addition of 7.5 µl of labelling reaction (2 µl Sequenase buffer, 2 µl Sequenase enzyme dilution buffer containing 1 unit of Sequenase version 2.0, 2 µl labelling mix, 1 µl 100 mM DTT, 0.5 µl ^{35}S dATP), incubated for 2

min at room temperature and terminated with 3.5 µl of standard dideoxynucleotides mixes containing 10% DMSO. The sequencing reactions, after the addition of stop mix (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) can be directly loaded onto an 8M sequencing gel. The results are shown in Figure 18 in which Lane 1 is the PCR product from a modified RNA template and Lane 2 is a DNA positive control template. Lane kb shows DNA size markers.

The RNA template was modified by one of five different acetylating agents. These were acetic anhydride, acetyl chloride, benzoic anhydride, trimethyl acetic anhydride and benzoyl chloride. Note that acetic anhydride and acetyl chloride both add the same group to the RNA ($-COCH_3$) as do benzoic anhydride and benzoyl chloride ($-COC_6H_6$). However different reactivities and specificity's are associated with each reagent and therefore the products cannot be considered equivalent. One of eight different thermostable enzymes or enzyme mixtures or preparations was used in a standard PCR assay. Results are summarised in Table 2.

Table 2. DNA template activity of modified RNA

Modification → / Enzyme ↓	DNA positive control	Acetic anhydride	Acetyl chloride	Benzoic anhydride	Benzoyl chloride	Trimethyl acetic anhydride
Taq	√	√	√	√	±	√
Taq/RNase A*	√	×	×	×	×	×
Tth	√	√	√	√	±	√
Tfl	√	√	√	√	±	√
Tli	×	×	×	×	×	×
Hot Tub (*Tfl*)	√	√	√	√	±	√

Key. √ **positive amplification.** × **no amplification.** ± **reduced amplification.**

*As a control for DNA contamination, samples were treated with RNase A (1µg) for 15 min at 37°C. This control confirmed that modified RNA was serving as the template because no PCR products were detected except with the DNA positive control template

Of note was the enhancement of PCR product yield from a modified RNA template when the annealing temperature was reduced from 65°C to 45°C for the initial 5 cycles of the PCR. The cycle conditions were either 94°C x 20 sec, 65°C x 20 sec and 72°C x 30 sec for 5 cycles and then 94°C x 20 sec, 55°C x 20 sec and 72°C x 30 sec for 25 cycles or 94°C x 20 sec, 45°C x 20 sec and 72°C x 30 sec for 5 cycles and then 94°C x 20 sec, 55°C x 20 sec and 72°C x 30 sec for 25 cycles. The enhancement is most likely a result of the improved annealing of the SP6 PCR primer with the modified RNA template at 45°C whilst at 65°C the annealed primer is unstable.

Reverse Transcription Results

Both Superscript II and HIV reverse transcriptases can copy modified RNA into a complementary DNA strand. However a large reduction in the amount of product (50-100 fold less than normal RNA) was observed with Superscript II reactions when acetylated RNA was reverse transcribed using oligo (dT). This reduction was probably due to the thermal instability of the oligo (dT): modified poly (A) RNA hybrid because modified RNA appears to have a reduced melting temperature. Effective priming was obtained using primers such as SP6 that contain G and C bases which increase stability. Of note is the increased incorporation (1.2 - 2.9 fold increase) of label using benzoic anhydride modified RNA compared with normal RNA. Gel analysis of the cDNA demonstrated a large proportion of full length cDNA generated from acetic anhydride modified RNA.

Conclusion

Modified RNA served as an effective polymerase template for several thermostable enzymes including *Taq*. PCR amplification was efficient without a prior reverse transcriptase step indicating that modified RNA is recognised by a DNA polymerase. One of the differences detected with modified RNA was the requirement for a primer containing G or C, the difference probably reflecting the reduced annealing temperature.

A PCR product derived from amplification of a DNA template or modified RNA template was purified and sequenced on both strands. No mutations were detected in over 600 bp of sequence analysed for each template type suggesting that if the amount of misincorporation increases by using modified RNA, it does so to a very low extent.

Advantages of Modified RNA Templates

In separate experiments, modified RNA has been shown to have greatly increased resistance to conditions that rapidly degrade RNA. Conditions necessary for the effective use of *Tth* and *Taq* enzymes (high temperature and cation concentration) are also optimal for the degradation of the template RNA. It would therefore be advantageous to be able to use conditions that were optimal for enzyme activity but did not lead to the degradation of the template. By modifying the 2'-OH groups, the modified RNA retains both its template activity and completeness with conditions where a substantial proportion of the normal RNA is degraded.

There are many kits, reagents and methods developed for the conversion of RNA into DNA prior to PCR. Kits are usually composed of RT and PCR components. A great advantage of the use of modified RNA is that no RT step is required; modified RNA can be added directly into the PCR reaction and amplified. The methods disclosed therefore are improved over prior methods for the reverse transcription of RNA.

The decreased melting temperature of the modified template RNA should also reduce the amount of secondary structure. RNA secondary structure leads to DNA polymerase obstruction and as a result chain termination and incomplete DNA copies. The secondary structure of mRNA is the major impediment to the production of full length cDNA clones and libraries. Unfortunately state of the art methods that have been developed to reduce the amount of secondary structure also result in RNA degradation. Because modified RNA has less secondary structure, enzyme obstruction should be reduced and therefore the proportion of full length cDNA clones increased. In addition, the template modified RNA is not becoming degraded and therefore the quality of the template is also improved.

PCR Amplification of Modified RNA Templates

The following one enzyme method was used to amplify modified RNA templates. To 0.1-1µg of RNA template containing SP6 and T3 sites, in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the

reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g.

Portions of the modified RNA (100 pg to 10 ng) were used as templates in a PCR. The PCR was carried out in a final volume of 25 µl with final concentration of 15 mM Tris-HCl pH 8.8, 60 mM KCl, 2.5 mM $MgCl_2$, 400 µM each dNTP, 10 pmol of each primer SP6 and T3 and 1 unit *Taq* DNA polymerase (Amersham, UK). Cycle parameters were 94°C x 20 sec, 55°C x 20 sec and 72°C x 30 sec for 30 cycles. PCR products were visualised following agarose gel electrophoresis and staining with EtBr.

RT-PCR Amplification of Modified RNA Templates

The following two enzyme method was used to amplify modified RNA templates. To 0.1-1µg of RNA template containing SP6 and T3 sites, in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl

(10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g.

Portions of the modified RNA (100 pg to 10 ng) were used as templates for reverse transcription with Superscript II enzyme. 100 ng (modified or normal) RNA was heated for 10 min at 75°C in 10 µl of water containing 50 ng SP6 primer and then left on ice. To this was added 2 µl of 25 mM $MgCl_2$, 2 µl 100 mM DTT, 1 µl 10 mM dNTPs, 1 µl 32P dCTP and 1 µl (10 units), Superscript II (Gibco-BRL, USA) (10 units). The reaction was incubated within the temperature range 37°C to 55°C for 30 minutes. Template was removed by incubating samples with RNase A (1µg) for 15 min at 37°C. 1 µl aliquots of the reverse transcription reaction were added to the following PCR mixture. The PCR was carried out in a final volume of 25 µl with final concentration of 15 mM Tris-HCl pH 8.8, 60 mM KCl, 2.5 mM $MgCl_2$, 400 µM each dNTP, 10 pmol of each primer SP6 and T3 and 1 unit *Taq* DNA

polymerase (Amersham, UK). Cycle parameters were 94°C x 20 sec, 55°C x 20 sec and 72°C x 30 sec for 30 cycles. PCR products were visualised following agarose gel electrophoresis and staining with EtBr.

Reverse Transcription with *Tth* DNA polymerase

Reverse transcription with *Tth* DNA polymerase offers the advantage of elevated reaction temperature that can reduce the amount of RNA secondary structure. The following two enzyme method was used to amplify modified RNA templates. To 0.1-1µg of RNA template containing SP6 and T3 sites, in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 4-dimethylaminopyridine (DMAP) and then 1 µl (10 µmoles) of acetic anhydride was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 60 seconds at room temperature (22°C). The reaction was then terminated by addition of three reaction volumes of ethanol or methanol followed by mixing with a vortex for 5 seconds. Modified RNA was removed from the reactants and solvent using one of several methods. The preferred method was to dilute the mixture into a final volume of 400 µl of water which was then added to a Centricon-50 spin-column (Amicon, USA) and centrifuged for 15 minutes at 3000g or until the filter was dry. The filter was then washed by addition of 400 µl of water and again spun for 15 minutes at 3000g. The modified RNA was recovered by inverting the cup containing the filter in a fresh centrifuge tube and spinning it for 60 seconds at 3000g.

The following protocol is essentially identical to the one provided by Boehringer Mannheim GmBH. To 2 µl of 1 X buffer (10 mM Tris-HCl pH 8.9, 90 mM KCl) was added 2 µl of 9 mM $MnCl_2$, 0.4 µl of 200 µM each dNTP, 750 nM SP6 primer, 50-200 ng modified template RNA and 1 µl (4 units) of *Tth* DNA polymerase and water to a final volume of 20 µl. The reaction was incubated for 30 min at 70°C. DNA products could then be used in a standard PCR reaction or visualised by adding trace quantities (1 µl) of radioactive ^{32}P dATP to the reaction and separating the products by gel electrophoresis.

Example 47

Hybridisation

Experimental Approach

Modified RNA was either immobilised onto a solid support such as a filter membrane (target) or labelled with radioactivity (probe) and allowed to hybridise with the target. Comparisons were made between modified and normal RNA as target and probes.

Dot blotting

RNA samples were labelled as follows. 100 ng of either modified (acetylated) or normal RNA was added to 13 µl of water, 2 µl 10 x kinase buffer and 1 µl of shrimp alkaline phosphatase (Boehringer Mannheim) added. The reaction was incubated for 10 min at 37°C and then the enzyme destroyed by treating for 10 min at 65°C. The 5′ end of the RNA was then labelled by the addition of 2.5 µl ^{32}P γ-ATP and 1 µl of T4 polynucleotide kinase (Boehringer Mannheim) and incubating 90 min at 37°C. Unincorporated label was

removed using a Centricon-50 column according to manufacturers instructions.

cDNA target was prepared using 1000 ng of 7.5 kb poly (A) tailed RNA (Cat. No. 15621-014, Gibco-BRL, USA) using a Superscript II cDNA kit (Gibco-BRL, USA) using oligo (dT) as a reverse transcriptase primer according to manufacturer's instructions. The reaction was terminated by incubating at 70°C for 10 minutes. RNA was removed by treatment with RNase H (200 ng RNase H added to the reaction and incubated 15 min at 37°C) and 50 ng of the remaining cDNA spotted onto a 5 mm square of Hybond N+ and allowed to dry before u/v cross-linking for 3 min. and baking at 65°C for 10 min. Two such squares were hybridised with either a modified or normal 7.5 kb RNA ^{32}P labelled probe at 65°C over-night in Church buffer. The squares were then washed at room-temperature in 1 x Church buffer and results quantitated by scintillation counting.

Figure 2 shows a comparison of the hybridisation properties of modified RNA and RNA in which Panel A is modified 7.5 kb RNA probe and panel B is normal 7.5 kb RNA probe. Each probe was hybridised to an immobilised cDNA target.

Comparison of different hybridisation membranes.

In order to select an optimum hybridisation membrane to be used, a portion of radiolabelled modified (acetylated) RNA was spotted onto 5 mm squares of six different membranes (Protran NC, Hybond N+ (Amersham, UK), Immobilon for DNA sequencing, Porablot NCL, Porablot PVDF, Immobilon P) and dried at room-temperature. each square was then washed twice for 5 min at 65°C in Church buffer and the amount of

radioactivity remaining on the squares quantitated using a scintillation counter.

Table 3.

Binding properties of different hybridisation membranes

Membrane	% CPM Remaining after washing
Protran-NC	24.4%
Hybond N+	31.6%
Immobilon	10.3%
Porablot NCL	20.7%
Porablot PVDF	19.8%
Immobilon P	6.4%

From these results it was apparent that Hybond N+ was the best membrane for binding acetylated RNA. However, Hybond N+ was less suitable than nitrocellulose for hybridisation. Hybridisation signals were approximately two times stronger when the modified RNA was attached to nitrocellulose than Hybond N+. Nitrocellulose membranes were therefore preferred for some applications.

A further comparison was made between modified (acetylated) RNA spotted onto membranes in a denatured or native (folded) state. Denaturation was brought about by heating at 68°C for 5 min in a 50% formamide/ 2.2 M formaldehyde solution prior to spotting on Hybond N+ membranes and hybridising with a labelled cDNA probe. No significant differences were detected in the hybridisation signals between denatured and native folded modified RNA.

Northern Blotting Procedure

Northern blotting was carried out according to Goda and Minton (1995) Nucleic Acid. Res. 16:3357-3358. Briefly, gels were prepared by adding 0.5 ml of 1 M guanidine thiocyanate and 2 µl of EtBr (10 mg/ml) in 100 ml of molten 1.2% agarose containing 1 x TBE buffer. Modified (acetylated) or normal RNA (0.24-9.5 kb RNA ladder (Cat. No. 15620-016, Gibco-BRL, USA); CAT mRNA, luciferase (Promega, USA) or human liver mRNA (Clontech, USA) was denatured by mixing a 10 µl sample (25 ng - 1 µg) with 10 µl formaldehyde and 5 µl formamide, heating at 90°C for 5 min. and then adding 10 x loading dye (50% glycerol, 1 mM EDTA, pH 8.0, 0.4% Bromophenol blue. Following electrophoresis at 100 V for 2 hrs the gel was photographed see panel A) and then the RNA was transferred to Hybond N+ (Amersham, UK) membrane according to manufacturers instructions. The membrane was hybridised overnight at 65°C in 'Church buffer' with a radioactive probe.

Deprotection with ammonia

Under the conditions used, the modified RNA hybridised only very weakly to the probe. Results are shown in Figure 20. By contrast the normal RNA gave a strong signal (panel B). By removing (deprotecting) the acetyl groups from the modified RNA using ammonia treatment, hybridisation was restored (panel C). The failure to hybridise may have been due to the reduction in Tm of the modified RNA or interaction between the charged carbonyl group (C=O) which is part of every acetyl group (-CO-CH3). The negative charge on the oxygen may be sufficient to allow interaction with the positive charges covering the Hybond N+ membrane, and as a result cause the modified RNA to adopt a

conformation not compatible with hybridisation. 50 ml of ammonium hydroxide (26%) was added to the northern membrane and incubated for 5 min. at room temperature. The membrane was rinsed with water and then immersed in Church buffer for 10 min. Hybridisation was carried out as described.

Figure 20 shows a comparison of modified and unmodified RNA behaviour on agarose gel and northern blotting. Panel A shows an EtBr stained agarose gel (lane 1) 0.24-9.5 kb RNA ladder (Cat. No. 15620-016, Gibco-BRL, USA), (lane 2) 0.24-9.5 kb RNA ladder modified by acetylation prior to electrophoresis. Note the differences in mobility and the increased degradation of normal RNA. Panel B shows that acetylated RNA does not hybridise appreciably to a radioactive cDNA probe when bound to a nylon membrane under standard conditions. Panel C shows that, following removal of the acetyl groups from the modified RNA by ammonia treatment, hybridisation is strong.

Change in Electrophoretic Mobility of Modified RNA

Figure 21 demonstrates the relationship between electrophoretic mobility (mm) and molecular weight (bases) of modified (acetylated) and normal RNA (see Panel A of Figure 20). The upper line represents unmodified RNA and the lower line represents modified RNA. Modified RNA migrates at approximately 75% the rate of normal RNA reflecting its increased molecular weight due to the acetyl group and possibly a change in secondary structure.

Conclusion

RNA modified by acetylation has altered hybridisation properties, probably reflecting a lower Tm of the hybrid.

Standard conditions of hybridisation for northern blotting are probably too stringent and a lower temperature should be chosen. Removal of the modifying groups reconstitutes the hybridisation properties of the RNA.

Significant advantages of the use of modified RNA for northern blotting are as follows. 1) Modified RNA binds to the hybridisation membrane with greater efficiency than normal RNA; 6 fold more modified RNA is retained on the membrane after washing in a strong detergent solution at 65°C than normal RNA. 2) Modified RNA does not degrade during electrophoresis and as a result it represents faithfully the starting material. 3) Simpler northern blotting materials can be used because the modified RNA has a reduced melting temperature. Without secondary structure formation, the RNA can be electrophoresed under mild conditions without the use of toxic denaturants such as formaldehyde. Despite their toxicity, formaldehyde northern blots are currently the standard procedure. The modified RNA provides much clearer separation of bands and unlike normal RNA, no degradation.

Example 48

Acylation in aqueous-tetrahydrofuran solution

To 0.1-1 µg of RNA in 1 µl of water was added 20 µl of tetrahydrofuran containing a catalytic quantity (60 µg; 490 nmoles) of DMAP and then 10 µmole of acetic anhydride or other acylating agent was added. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 20-60 minutes at room temperature before it was terminated by the addition of three volumes of ethanol and mixing. The modified RNA could be purified

away from the propanoic, butyric, pentanoic, hexanoic, heptanoic, octanoic or benzoic anhydrides.

Example 49

Acylation in aqueous-dimethyl formamide solution using 4-pyrrolidinopyridine

To 0.1-1 µg of RNA in 1 µl of water was added 20 µl of dimethyl formamide containing a catalytic quantity of 4-pyrrolidinopyridine and then 0.1-10 µmole of acetic anhydride or other acylating agent was added. The preferred reaction contained 1 µg of RNA, 20 µl of dimethyl formamide, 60 µg of 4-pyrrolidinopyridine and 0.1 µmole of acetic anhydride. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 20-60 minutes at room temperature before it was terminated by the addition of three volumes of athanol and mixing. The modified RNA could be purified away from the reactants using Centricon-50 spin-column or ethanol precipitation. Other acylation agents include benzoic, propanoic, butyric, valeric, caproic, oenanthic and caprylic anhydrides.

Example 50

Asylation in aqueous-dimethyl formamide solution using DMAP

To 0.1-1 µg of RNA in 1 µl of water was added 20 µl of dimethyl formamide containing a catalytic quantity (60 µg; 490 nmoles) of DMAP and then 0.1-10 µmole of acetic anhydride or other acylating agent was added. The preferred reaction contained 1 µg of RNA, 20 µl of dimethyl formamide, 60 µg of DMAP and 1µmole of acetic anhydride. The solution was mixed vigorously using a vortex for 5

seconds and the reaction allowed to proceed for 20-60 minutes at room temperature before it was terminated by the addition of three volumes of ethanol and mixing. The modified RNA could be purified away from the reactants using Centricon-50 spin-column or ethanol precipitation. Other acylation agents include propanoic, butyric, pentanoic, hexanoic, heptanoic, octanoic or benzoic anhydrides.

Example 51

Acylation using 2-hydroxypyridine catalyst

To 0.1-1 µg of RNA in 1µl of water was added 20 µl of triethylamine containing a catalytic quantity (60 µg; 490 nmoles) of 2-hydroxypyridine and then 0.1-10 µmole of acetic anhydride or other acylating agent was added. The preferred reaction contained 1 µg of RNA, 20 µl of TEA, 60 µg of DMAP and 1 µmole of acetic anhydride. The solution was mixed vigorously using a vortex for 5 seconds and the reaction allowed to proceed for 5-20 minutes at room temperature before it was terminated by the addition of three volumes of ethanol and mixing. The modified RNA could by purified away from the reactants using Centricon-50 spin-column or ethanol precipitation. Other acylation agents include propanoic, butyric, pentanoic, hexanoic, heptanoic, octanoic or benzoic anhydrides.

EXAMPLE 52

Use of tetraethylammonium acetate for acetylation

To 1 µg (1.7 pmol) of mRNA was added 1 µmol of tetraethylammonium acetate and the mixture rendered anhydrous and then resuspended in 1 µl (10 µmol) of acetic

anhydride. Following an incubation period of 2 hrs at room temperature, 10 µl of 1:1 (v:v) pyridine;water was added and the reaction incubated 5 hrs at room temperature. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) of Microcon-50 spin-column purification (Amicon, USA) as described.

Example 53

Use of pyridinium chlorochromate for oxidation

The oxidation of alcohols (-OH->=O) is a well known procedure and pyridinium chlorochromate (Corey's Reagent) is particularly useful as an oxidising agent. The use of acetic acid can improve the reaction rate (Agarwal et al, (1990) Tetrahedron 46:4417-4420). To 1 µg (1.7 pmol) of mRNA in 10 µl of dimethyl formamide was added 5 nmol of pyridinium chlorochromate, 40 µl (1.6 nmol) of acetic acid to serve as an acid catalyst and the mixture incubated for 2 hrs at room temperature. The unreacted components were removed either by ethanol precipitation (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) or Microcon-50 spin-column purification (Amicon, USA) as described.

EXAMPLE 54

Stability of RNA modified with butyric and pentanoic anhydrides.

RNA was modified with either butyric or pentanoic anhydrides in accordance with the method of example 6.

Figure 22 shows enhanced stability of RNA modified with butyric and pentanoic anhydrides. The Lanes are as follows: Lane 1; Radiolabelled riboprobe (Promega, USA) RNA, lane 2; butyric anhydride modified RNA, lane 3; pentanoic anhydride modified RNA, lanes 4-6; samples treated with 5 units of mung bean nuclease for 10 minutes at 37°C, lane 4; RNA, lane 5; butyric anhydride modified RNA, lane 6; pentanoic anhydride modified RNA, lanes 7-9; samples treated with 15 units of S1 nuclease for 10 minutes at 37°C, lane 7; normal RNA, lane 8; butyric anhydride modified RNA, lane 9; pentanoic anhydride modified RNA, lanes 10-12; samples treated in 80mM NaOH for 15 minutes at 22°C, lane 7; normal RNA, lane 9; butyric anhydride modified RNA, lane 9; pentanoic anhydride modified RNA, land 13; marker lane with acetic anhydride modified RNA. Note the complete degradation of RNA with mung bean nuclease (lane 4), S1 nuclease (lane 7) and its partial degradation with alkali (lane 10) compared with modified forms.

Example 55

Acetyl chloride modification of RNA in presence and absence of catalyst

This example compares with the degree of RNA acetylation as set out below in accordance with the method of example 8 in the presence and absence of the catalyst, DMAP.

Figure 23 shows a comparison of the degree of RNA acetylation using different concentrations of acetyl chloride with or without the catalyst DMAP. The lanes are as follows: Lane 1; radiolabelled riboprobe RNA marker (Promega, USA), lane 2, acetylated riboprobe RNA marker,

Lanes 3-12; acetylation of RNA with acetyl chloride in a catalysed (3mg/ml DMAP) (lanes 3-7) or uncatalysed (lanes 8-12) solvent system. Acetyl chloride of varying concentration and in a final volume of 1 µl of toluene was mixed with 100 ng RNA in 1 µl of water, 20 µl of TEA with or without a catalyst and incubated for 20 seconds at room temperature. Lane 3; 0.25%, lane 4; 0.5%, lane 5; 0.75%, lane 6; 1% and lane 7; 1.25% final concentration of acetyl chloride with DMAP. Lane 8; 0.25% , lane 9; 0.5%, lane 1-; 0,75%, lane 11; 1% and lane 12; 1.25% final concentration of acetyl chloride without DMAP. The amount of acetylation increases with increasing acetyl chloride concentration and in the presence of DMAP catalyst. Even when the highest concentrations of acetyl chloride are used with the catalyst (lane 7), there is less modification than with acetic anhydride (lane 2).

EXAMPLE 56

Acetylation of RNA with prolonged reaction time.

Using the methology of example 37, this example shows the effect of reaction time on acetylation of RNA using acetic anhydride.

Figure 24 shows increased acetylation of RNA when reaction times are prolonged. Modification reactions were carried with 20 ng of radiolabelled riboprobe RNA and 100 ng yeast RNA, 1 µl of acetic anhydride, 20 µl of TEA containing 3 mg/ml of DMAP, mixed and incubated for the following times. The lanes are as follows: Lane 1; 0 seconds, lane 2; 20 seconds, lane 3; 1 minute, lane 4; 6 minutes and lane 5; 20 minutes at room temperature before the reaction was stopped

and analysed on a sequencing gel. Reactions were stopped by the addition of three volumes of ethanol with mixing.

EXAMPLE 57

Extent of RNA modification using DMAP catalyst

This example compares the extent of RNA modification in the presence and absence of the catalyst DMAP. A DMAP catalysed acetylation reaction was carried out in accordance with example 6 and compared with an analogous reaction carried out in the absence of DMAP in accordance with example 36.

Figure 25 shows that catalyst DMAP increases the amount of RNA modification by acetic anhydride. Lane 1; 20 ng of radiolabelled riboprobe RNA, 10 µg of yeast RNA, 10 µg of acetic anhydride and 20 µl of TEA containing 3 mg/ml of DMAP, lane 2; 20 ng of radiolabelled riboprobe RNA, 10 µl of yeast RNA, 1 µl of acetic anhydride, 20 µl of TEA with no DMAP, lane 3; unmodified RNA size marker. Reactions were carried out for 20 seconds at room temperature. Note the distinct step between lanes 1 and 2 demonstrating that the RNA in lane 2 is more modified in the presence of DMAP than without (lane 1).

COMPARATIVE EXAMPLE

An attempt was made to reproduce the work of OVODOV and ALAKHOV (1990) FEBS 270: 111 who report acetylation of 70-75% of the 2'- OH groups of a mRNA from a cell-free transcription system using the acetylation method of KNORRE et al (1967) Molekul.Biol 1: 837. The results of the Knorre method were compared with the results from the methods according to the present invention.

Figure 26 shows a comparison of the efficacy of the two acetylation methods. Radiolabeled RNA ladders derived from an *in vitro* transcription reaction (Promega, USA) were treated with acetic anhydride either in an aqueous-DMF solvent system according to Knorre et al (Lanes 1-11) or in a 19:1 TEA:aqueous with a DMAP catalyst (lane 12) according to example 6 of the present application. For clarity, only one labelled RNA marker is shown. A decrease in mobility reflects the increase in molecular weight of the RNA indicating a successful modification reaction. The Lanes are as follows:Lane 1, Ribomark RNA treated for 2 hrs at 37°C and then 46 hrs at room temperature, lane 2, Ribomark RNA treated for 48 hrs at room temperature, unmodified Ribomark RNA (lanes 3 and 130, Ribomark RNA treated for 1 hr at 4°C with 0.01 µl(lane 4), 0.1 µl (lane 6) or 10 µl (lane 7) of acetic anhydride. Ribomark RNA treated for 1 hr at 37°C with 0.01 µl (lane 8), 0.1 µl (lane 9), 1 µl (lane 11) of acetic anhydride. Lane 12, Ribomark RNA treated according to example 6.

It was found that the condition according to Knorre were quite unable to modify the RNA even when reaction times were extended from the 1 hour specified to 48 hours (lanes 1 and 2) or acetic anhydride concentrations were increased 1000 times from the 98 nmol specified (lanes 4 and 8) to 98 µmol per 1 µg of RNA or reaction temperatures were increased from 4°C (lanes 407) to 37°C (lanes 8-11). In every case for the Knorre method, the RNA migrated at the same position as the unmodified controls (lanes 3 and 13). Only the TEA/DMAP/aqueous solvent system as described in

present example 6 resulted in modification (lane 12). This and further attempts to repeat the work of Ovodov and Alakhov failed, leading to the conclusion that the publication by Ovodov and Alakhov does not enable modification of RNA in the manner they describe. This finding is consistent with the results presented by Ovodov and Alakhov in their publication where the molecular weight of modified material is unchanged as compared with unmodified material.

CLAIMS

1. A polynucleotide comprising mRNA or viral RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position.

2. A polynucleotide according to claim 1, wherein at least 90% of the ribose rings are covalently modified at the 2'-OH position.

3. A polynucleotide according to claim 1 or claim 2, wherein the 2'-OH position of the ribose rings is covalently modified so that a single strand of the polynucleotide is replicable by a nucleic acid polymerase to generate a second strand of polynucleotide complementary to the single strand.

4. A polynucleotide according to any one of claims 1 to 3, wherein at least some of the modified ribose rings bear at the 2'-OH position a substituent which is labelled with a label.

5. A polynucleotide according to claim 4, wherein the label comprises a fluorescent label, a radioactive label, an enzyme, a ligand or an affinant for a label.

6. A polynucleotide according to any one of the preceding claims, wherein the modified ribose rings bear at the 2'-OH position a substituent, OR, wherein R is selected from: C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, C_1-C_{10}, alkoxyalkyl, C_1-C_{10}

haloalkoxyalkyl, C_1-C_{10} aminoalkoxyalkyl, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl, C_6-C_{14} arylalkenyl, C_1-C_{10} alkanoyl, C_1-C_{10} alkenoyl, C_1-C_{10} haloalkanoyl, C_1-C_{10} aminoalkanoyl, C_6-C_{14} arylalkanoyl, C_6-C_{14} arylalkenoyl, C_6-C_{14} aryloxyalkanoyl, C_6-C_{14} alkylarylalkanoyl, C_6-C_{14} haloarylalkanoyl, C_6-C_{14} aminoarylalkanoyl, C_1-C_{10} alkylsilanyl, C_{12}-C_{28} diarylphosphone; or a substituent, R', wherein R' is selected from C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, halo, amino, C_1-C_{10} alkylamino, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl.

7. A polynucleotide according to claim 6, wherein R is selected from: methyl, ethyl, vinyl, allyl, ethynyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, (2-chloroethyl)oxyethyl, (2-aminoethyl)oxyethyl, phenyl, 4-methylphenyl, benzyl, cinnamyl, acetyl, propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl, octanoyl, pivaloyl, 4-aminobutanoyl, 4-chlorobutanoyl, trifluoroacetyl, trichloroacetyl, acryloyl, propioloyl, crotonoyl, benzoyl, diphenylacetyl, phenoxyacetyl, 4-methylbenzoyl, 4-chlorobenzoyl, 4-aminobenzoyl, 4-nitrobenzoyl, cinnamoyl, silanyl, trimethylsilanyl, t-butyldimethylsilanyl, 2-chlorophenyl(4-nitrophenyl)phosphono; and R' is selected from methyl, ethyl, vinyl, allyl, ethynyl, t-butyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, phenyl, benzyl, fluoro, chloro, amino, keto.

8. A polynucleotide according to any one of the preceding claims, wherein the mRNA or viral RNA is naturally-occuring.

9. A polynucleotide according to any one of the preceding claims, wherein the mRNA or viral RNA comprises cellular RNA.

10. A mixture of polynucleotides comprising a mixture of cellular mRNA or viral RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position.

11. A mixture of polynucleotides according to claim 10, wherein each polynucleotide comprises a polynucleotide according to any one of claims 2 to 9.

12. A process for producing a modified oligo- or polynucleotide, which comprises (i) contacting in a reaction medium RNA comprising an oligo- or poly-ribonucleotide with a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA; (ii) reacting the RNA with the reactant to produce modified oligo- or polynucleotide under conditions to achieve covalent modification of greater than 75% of the 2'-OH positions of the ribose rings; and (iii) optionally separating the modified oligo- or polynucleotide from the reaction medium, wherein the reaction medium comprises at least 20% v/v organic solvent.

13. A process according to claim 12, wherein the reaction conditions are such that at least 90% of the ribose rings are covalently modified at the 2'-OH position.

14. A process according to claim 12 or claim 13, wherein the 2'-OH position of the ribose rings is covalently modified so that a single strand of the oligo- or polynucleotide is replicable by a nucleic acid polymerase to generate a second strand of a polynucleotide complementary to the single strand.

15. A process according to any one of claims 12 to 14, wherein at least some of the reactant is labelled with a label.

16. A process according to claim 15, wherein the label comprises a fluorescent label, a radioactive label, an enzyme, a ligand or an affinant for a label.

17. A process according to any one of claims 12 to 16, wherein the reactant is chosen such that the modified ribose rings bear at the 2'-OH position a substituent, OR, wherein R is selected from: C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, C_1-C_{10}, alkoxyalkyl, C_1-C_{10} haloalkoxyalkyl, C_1-C_{10} aminoalkoxyalkyl, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl, C_6-C_{14} arylalkenyl, C_1-C_{10} alkanoyl, C_1-C_{10} alkenoyl, C_1-C_{10} haloalkanoyl, C_1-C_{10} aminoalkanoyl, C_6-C_{14} arylalkanoyl, C_6-C_{14} arylalkenoyl, C_6-C_{14} aryloxyalkanoyl, C_6-C_{14} alkylarylalkanoyl, C_6-C_{14} haloarylalkanoyl, C_6-C_{14} aminoarylalkanoyl, C_1-C_{10} alkylsilanyl, C_{12}-C_{28} diarylphosphone; or a substituent, R', wherein R' is selected from C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, halo, amino, C_1-C_{10} alkylamino, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl.

18. A process according to claim 17, wherein R is selected from: methyl, ethyl, vinyl, allyl, ethynyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, (2-chloroethyl)oxyethyl, (2-aminoethyl)oxyethyl, phenyl, 4-methylphenyl, benzyl, cinnamyl, acetyl, propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl, octanoyl, pivaloyl, 4-aminobutanoyl, 4-chlorobutanoyl, trifluoroacetyl, trichloroacetyl, acryloyl, propioloyl, crotonoyl, benzoyl, diphenylacetyl, phenoxyacetyl, 4-methylbenzoyl, 4-chlorobenzoyl, 4-aminobenzoyl, 4-nitrobenzoyl, cinnamoyl, silanyl, trimethylsilanyl, t-butyldimethylsilanyl, 2-chlorophenyl(4-nitrophenyl)phosphono; and R' is selected from methyl, ethyl, vinyl, allyl, ethynyl, t-butyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, phenyl, benzyl, fluoro, chloro, amino, keto.

19. A process according to claim 17 or claim 18, wherein the reactant comprises an acid anhydride, an acid chloride, a carboxylic acid or an N-acylimidazole.

20. A process according to claim 19, wherein the reaction medium further comprises an acylation catalyst.

21. A process according to claim 20, wherein the RNA is reacted with the acid anhydride and the acylation catalyst comprises a fluoride ion or aminopyridine catalyst.

22. A process according to claim 20, wherein the RNA is reacted with the acid chloride and the acylation catalyst comprises an aminopyridine catalyst.

23. A process according to claim 20, wherein the RNA is reacted with the N-acylimidazole and the acylation catalyst comprises an aminopyridine catalyst.

24. A process according to any one of claims 12 to 23, wherein the reaction medium further comprises water.

25. A process according to claim 24, wherein the water and the organic solvent form two different phases in the reaction medium.

26. A process according to claim 25, wherein the RNA is reacted with the reactant under conditions of phase transfer catalysis.

27. A process according to claim 24 or claim 25, wherein the weight ratio of water:organic solvent is in the range 1:50 to 1:10.

28. A process according to claim 19, wherein the reactant comprises a carboxylic acid in the presence of a dehydrating agent or an isocyanide catalyst.

29. A process according to claim 17 or claim 18, wherein the reactant comprises an *O*-silylation agent.

30. A process according to claim 29, wherein the RNA is reacted with the *O*-silylation agent in the presence of an aminopyridine or lithium sulphide catalyst.

31. A process according to any one of claims 12 to 30, wherein the organic solvent comprises an organic base.

32. A process according to claim 31, wherein the organic base is the organic solvent.

33. A process according to any one of claims 12 to 32, wherein the reaction conditions are such that the covalent modification of the 2'-OH positions of the ribose rings is substantially regiospecific.

34. A process according to any one of claims 12 to 33, which further comprises prior to step (i) a step of protecting the exocyclic amino groups of the bases of the RNA with a protecting group; and after step (ii) a step of deprotecting the exocyclic amino groups by removing the protecting group.

35. A process according to claim 34, wherein the protecting group is benzoyl, *N*-phenoacetyl or *N,N*-dimethylaminomethylene for adenine; benzoyl for cytosine; and isobutyl, *N*-phenoacetyl or *N,N*- dimethylaminomethylene for guanine.

36. A process according to any one of claims 12 to 35, wherein the RNA comprises mRNA or viral RNA.

37. A process according to any one of cliams 12 to 36, wherein the RNA is attached to a solid phase.

38. A process according to any one of claims 12 to 37, wherein the RNA comprises an RNA sample from a cell or blood extract.

39. A method for gene expression analysis which comprises obtaining a polynucleotide comprising an mRNA sample modified in accordance with the process of claim 38 and analysing the polynucleotide.

40. A method for the replication of a polynucleotide, which comprises obtaining according to any one of claims 1 to 11 or by a process according to any one of claims 12 to 38, a polynucleotide comprising modified RNA, and replicating the modified RNA to form a complementary polynucleotide using a nucleic acid polymerase.

41. A method according to claim 40, wherein the complementary polynucleotide comprises a cDNA and the nucleic acid polymerase comprises a DNA polymerase.

42. A method according to claim 40 or claim 41, which further comprises a step of ligating to a vector a single or double stranded polynucleotide comprising the polynucleotide and the complementary polynucleotide.

43. A method for protecting and deprotecting RNA which comprises providing RNA; producing from the RNA a modified oligo- or poly-nucleotide according to the process of any one of claims 12 to 38 in which the 2'-OH position of greater than 75% of the ribose rings is modified with a substituent, and reacting the modified oligo- or poly-nucleotide with a reactant capable of removing the substituent under conditions to reinstate an -OH group at the 2'-OH position.

44. Use of an oligo- or poly-nucleotide comprising RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position, in a nucleic acid sequencing method.

45. Use of an oligo- or poly-nucleotide comprising RNA, greater than 75% of the ribose rings of which are covalently modified at the 2'-OH position, for the preparation of a medicament for binding specifically to an in vivo target to achieve a therapeutic, prophylactic or diagnostic effect.

46. Use according to claim 44 or claim 45, wherein the oligo or poly- nucleotide comprises a polynucleotide according to any one of claims 2 to 9.

47. A polynucleotide according to claim 1, obtainable by a process according to any one of claims 12 to 38.

48. A kit for modifying an oligo- or polynucleotide comprising an oligo- or poly-ribonucleotide, which kit comprises

(a) an organic solvent; and

(b) a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the oligo- or poly-ribonucleotide in the presence of the organic solvent.

49. A kit according to claim 48, wherein the reactant is chosen such that the modified ribose rings bear at the 2'-OH position a substituent, OR, wherein R is selected from: C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, C_1-C_{10}, alkoxyalkyl, C_1-C_{10}

haloalkoxyalkyl, C_1-C_{10} aminoalkoxyalkyl, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl, C_6-C_{14} arylalkenyl, C_1-C_{10} alkanoyl, C_1-C_{10} alkenoyl, C_1-C_{10} haloalkanoyl, C_1-C_{10} aminoalkanoyl, C_6-C_{14} arylalkanoyl, C_6-C_{14} arylalkenoyl, C_6-C_{14} aryloxyalkanoyl, C_6-C_{14} alkylarylalkanoyl, C_6-C_{14} haloarylalkanoyl, C_6-C_{14} aminoarylalkanoyl, C_1-C_{10} alkylsilanyl, C_{12}-C_{28} diarylphosphone; or a substituent, R', wherein R' is selected from C_1-C_{10} alkyl, C_1-C_{10} alkenyl, C_1-C_{10} alkynyl, C_1-C_{10} haloalkyl, C_1-C_{10} aminoalkyl, halo, amino, C_1-C_{10} alkylamino, C_6-C_{14} aryl, C_6-C_{14} alkylaryl, C_6-C_{14} arylalkyl.

50. A kit according to claim 49, wherein R is selected from: methyl, ethyl, vinyl, allyl, ethynyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, (2-chloroethyl)oxyethyl, (2-aminoethyl)oxyethyl, phenyl, 4-methylphenyl, benzyl, cinnamyl, acetyl, propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl, octanoyl, pivaloyl, 4-aminobutanoyl, 4-chlorobutanoyl, trifluoroacetyl, trichloroacetyl, acryloyl, propioloyl, crotonoyl, benzoyl, diphenylacetyl, phenoxyacetyl, 4-methylbenzoyl, 4-chlorobenzoyl, 4-aminobenzoyl, 4-nitrobenzoyl, cinnamoyl, silanyl, trimethylsilanyl, t-butyldimethylsilanyl, 2-chlorophenyl(4-nitrophenyl)phosphono; and R' is selected from methyl, ethyl, vinyl, allyl, ethynyl, t-butyl, 2-chloroethyl, 2-aminoethyl, ethyloxyethyl, phenyl, benzyl, fluoro, chloro, amino, keto.

51. A kit according to claim 49 or claim 50, wherein the reactant comprises an acid anhydride, an acid chloride, a carboxylic acid or an N-acylimidazole.

52. A kit according to claim 51, whrein the reaction medium further comprises an acylation catalyst.

53. A kit according to claim 52, wherein the reactant comprises the acid anhydride and the acylation catalyst comprises a fluoride ion or aminopyridine catalyst.

54. A kit according to claim 52, wherein the reactant comprises the acid chloride and the acylation catalyst comprises an aminopyridine catalyst.

55. A kit according to claim 52, wherein the reactant comprises the N-acylimidazole and the acylation catalyst comprises an aminopyridine catalyst.

56. A kit according to claim 51, wherein the reactant comprises a carboxylic acid in the presence of a dehydrating agent or an isocyanide catalyst.

57. A kit according to claim 49 or claim 50, wherein the reactant comprises an *O*-silylation agent in the presence of an aminopyridine or lithium sulphide catalyst.

58. A kit according to any one of claims 48 to 57, wherein the organic solvent comprising an organic base.

59. A kit according to claim 58, wherein the organic base is the organic solvent.

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RNA
Chain
5'
Base
O
4'
1'
3'
2'
OH
RNA
Chain
DNA
Chain
5'
Base
O
4'
1'
3'
2'
H
DNA
Chain

FIGURE 1a

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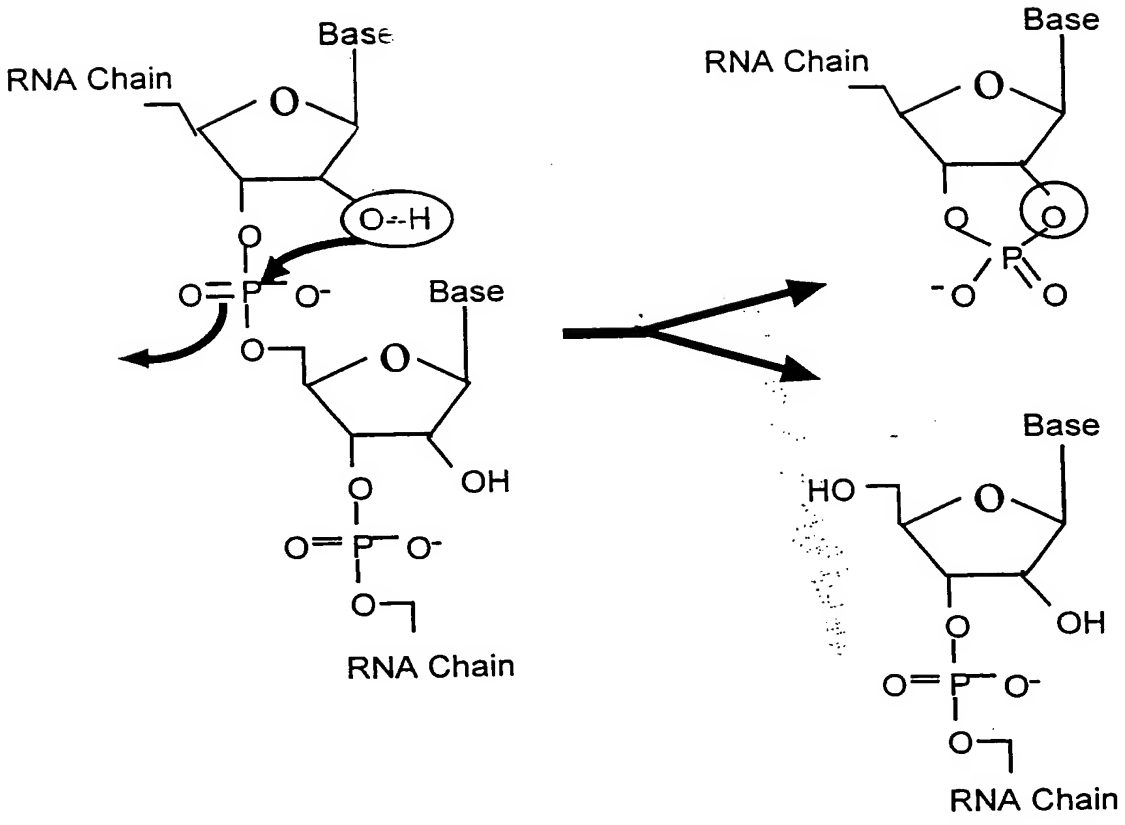

Base
RNA Chain
O
O-H
O
O=P-O-
Base
O
O
OH
O
O=P-O-
O
RNA Chain
Base
RNA Chain
O
O
O
P
-O
O
Base
HO
O
OH
O
O=P-O-
O
RNA Chain

FIGURE 1b

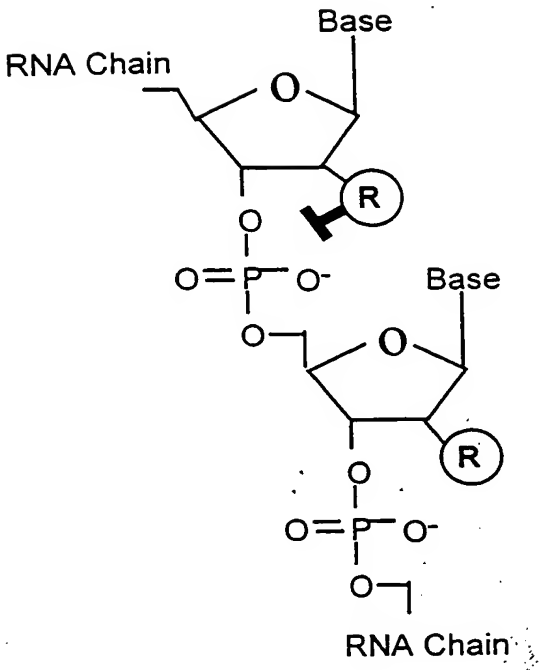


FIGURE 2

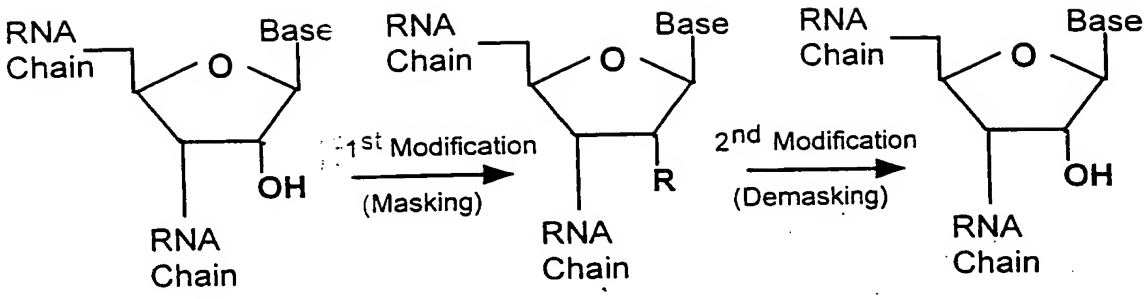

RNA Chain
Base
O
OH
RNA Chain
1st Modification
(Masking)
RNA Chain
Base
O
R
RNA Chain
2nd Modification
(Demasking)
RNA Chain
Base
O
OH
RNA Chain

FIGURE 3

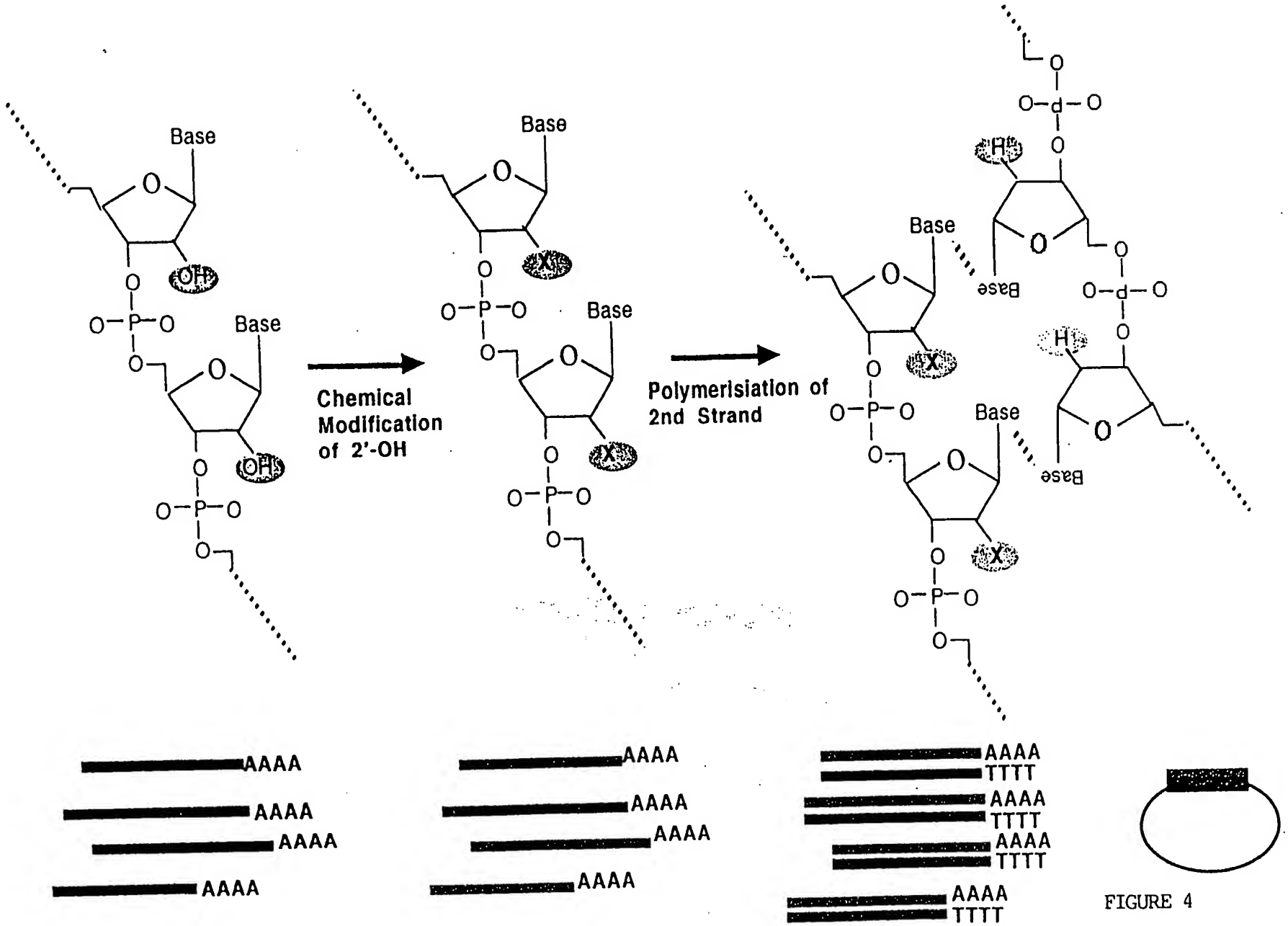

Base
OH
Chemical
Modification
of 2'-OH
X
Polymerisation of
2nd Strand
H
AAAA
TTTT

FIGURE 4

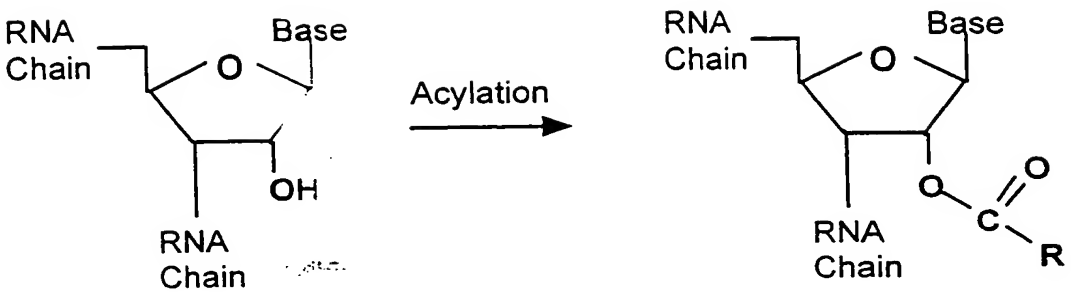

RNA
Chain
Base
O
OH
RNA
Chain
Acylation
RNA
Chain
Base
O
O
C
O
R
RNA
Chain

FIGURE 5a

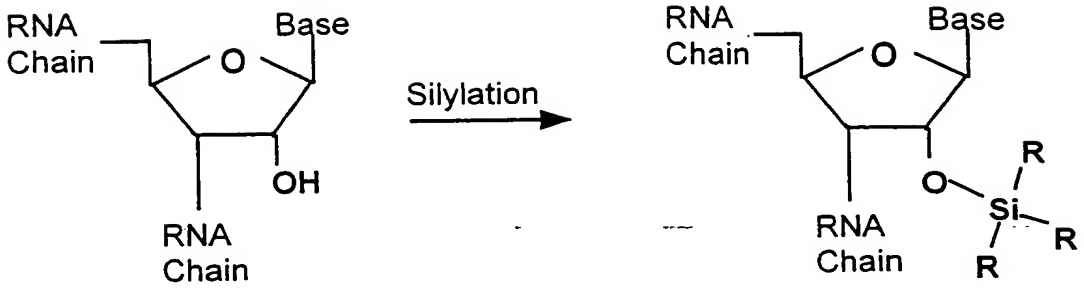

RNA
Chain
Base
O
OH
RNA
Chain
Silylation
RNA
Chain
Base
O
O
Si
R
R
R
RNA
Chain

FIGURE 5b

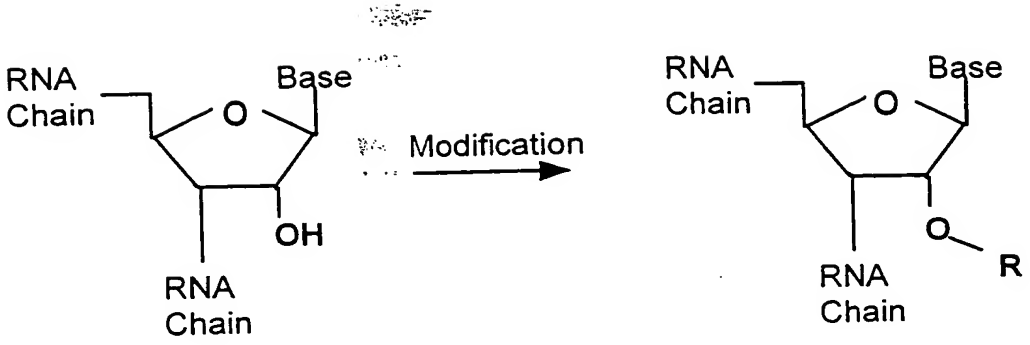

RNA
Chain
Base
O
OH
RNA
Chain
Modification
RNA
Chain
Base
O
O
R
RNA
Chain

FIGURE 5c

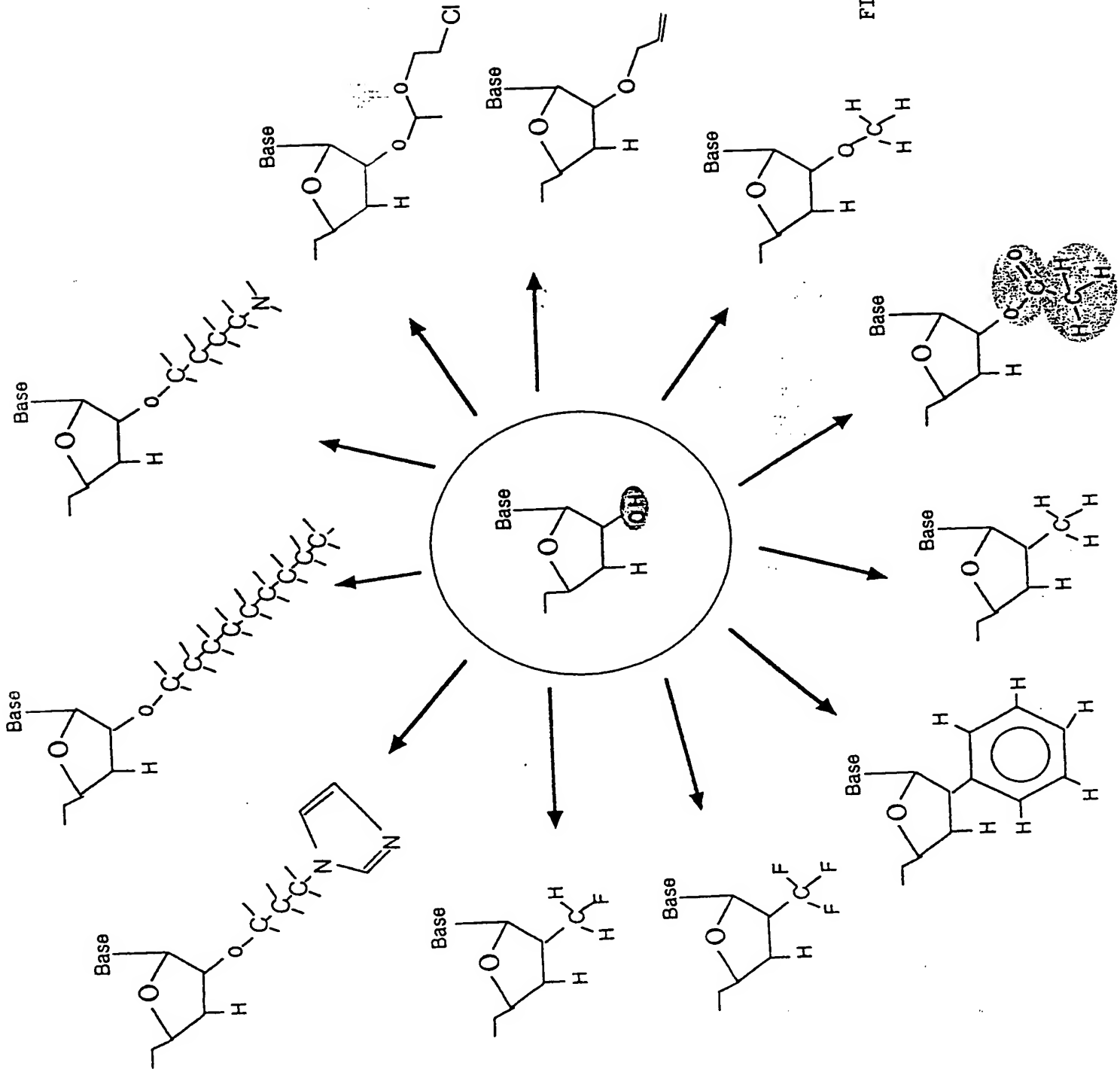


FIGURE 6

Example 7

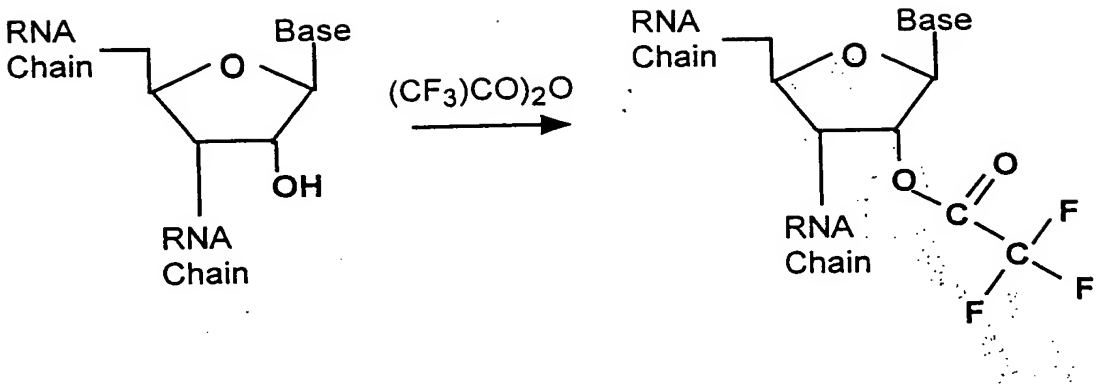


FIGURE 7

Examples 11 and 27 (formation of 2' chloro)

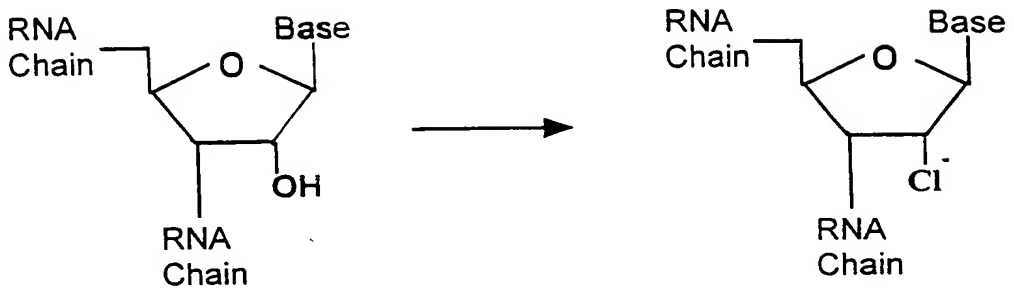
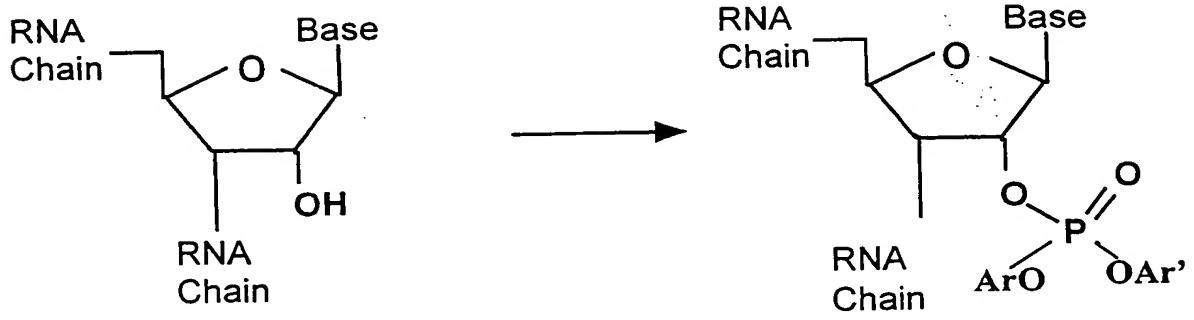


FIGURE 8

Example 12



Ar = 2-ClC_6H_4; Ar' = 4-$O_2NC_6H_4$

FIGURE 9

Example 13 (formation of 2' levulinate)

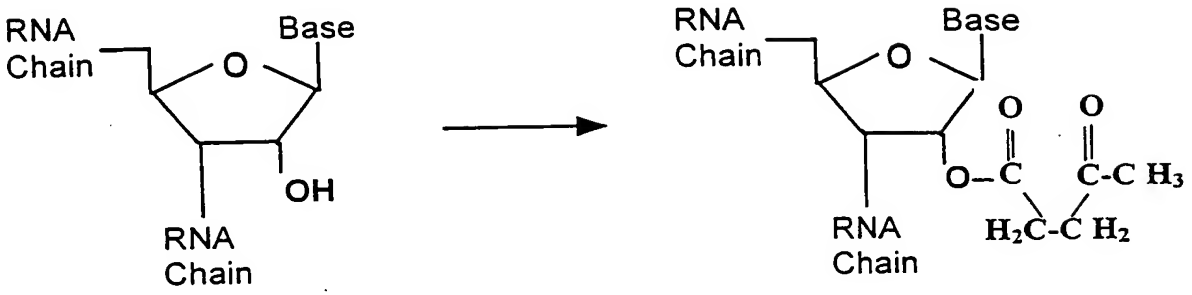


FIGURE 10

Example 14 (formation of 2' benzoyl)

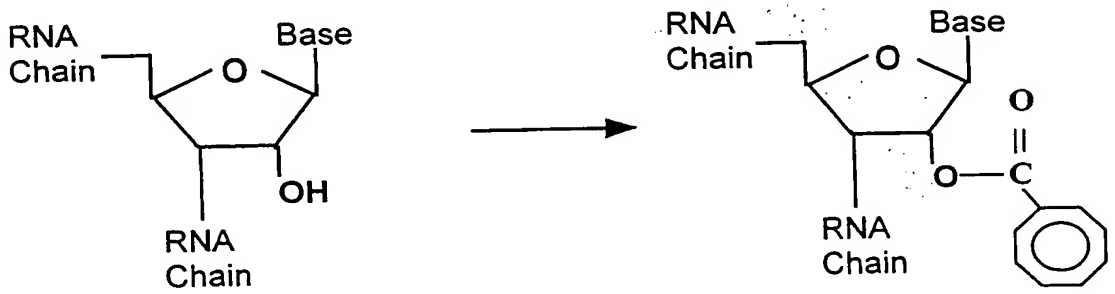


FIGURE 11

Example 19

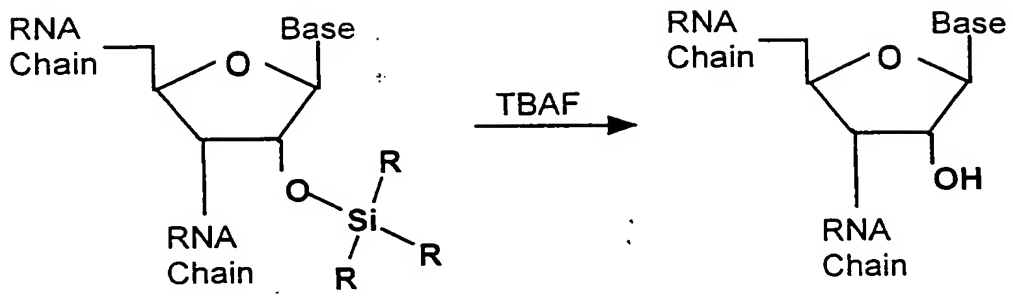


FIGURE 12

Example 21

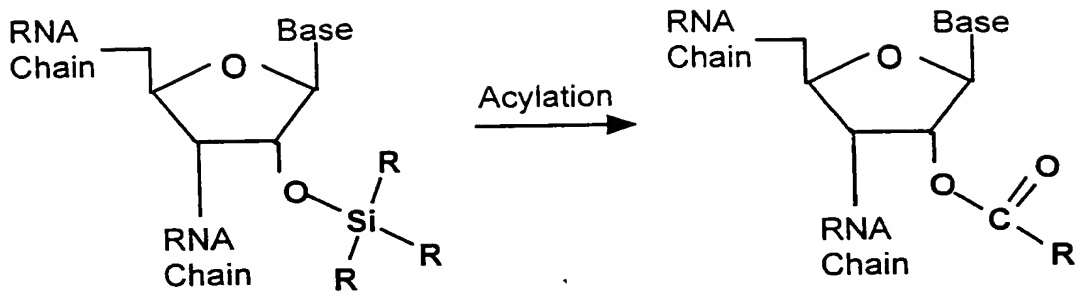


FIGURE 13

Example 29

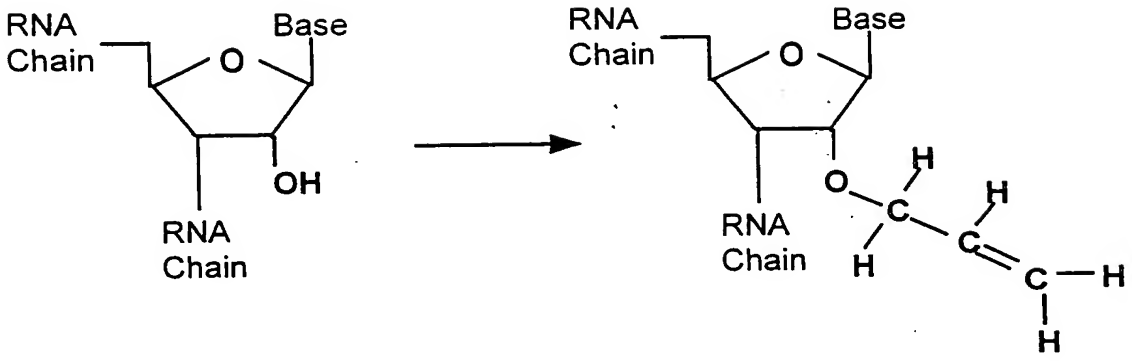


FIGURE 14

Example 30

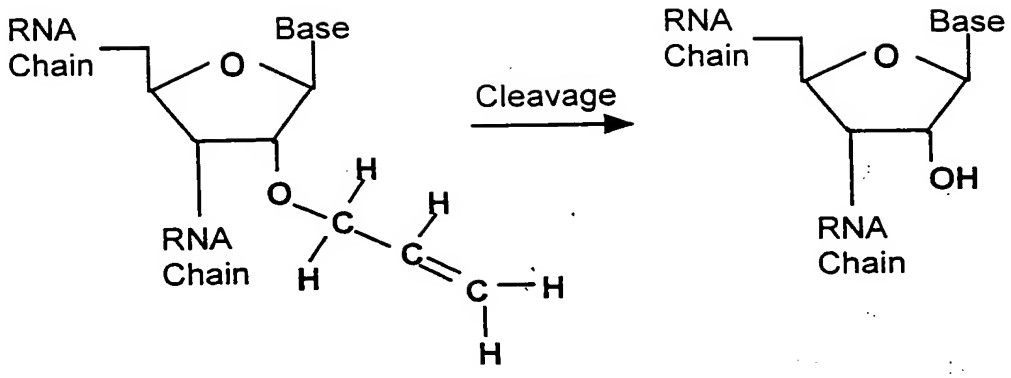


FIGURE 15

FIGURE 16

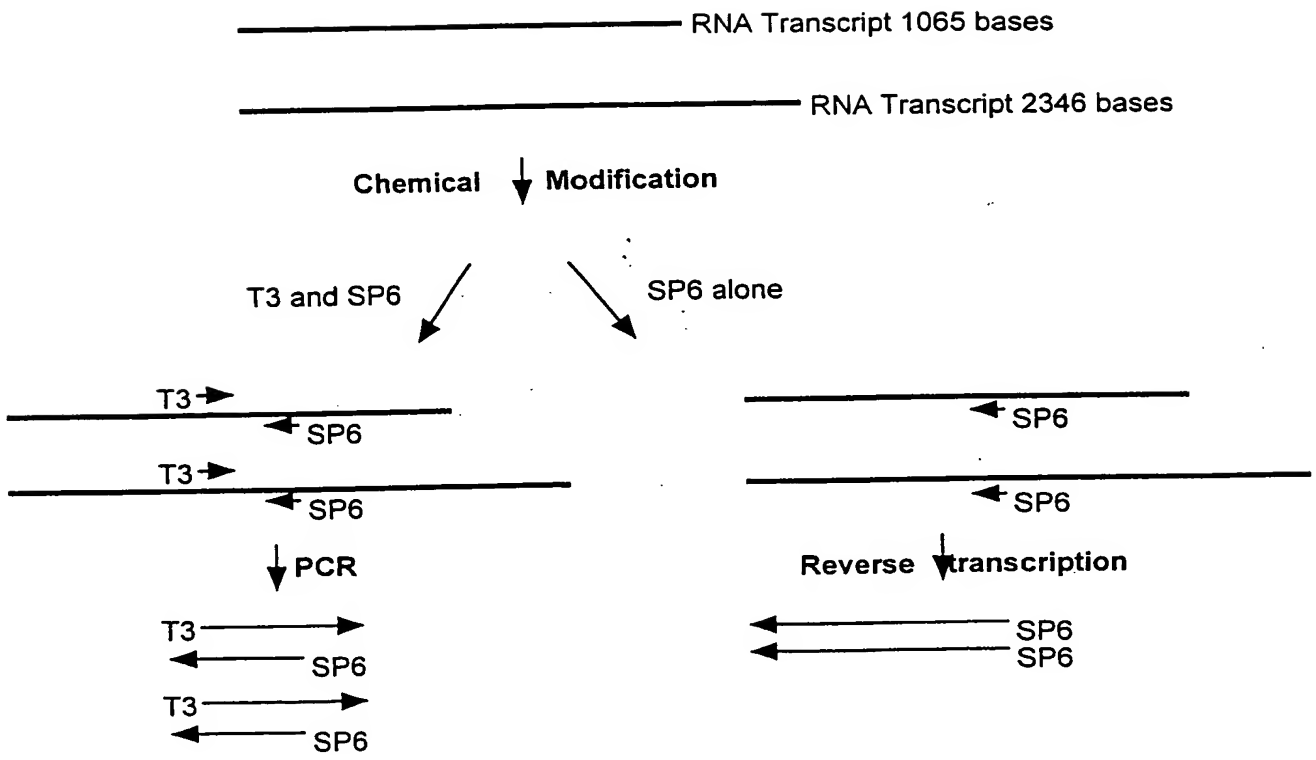

RNA Transcript 1065 bases
RNA Transcript 2346 bases
Chemical Modification
T3 and SP6
SP6 alone
T3
SP6
T3
SP6
PCR
T3
SP6
T3
SP6
SP6
SP6
Reverse transcription
SP6
SP6

FIGURE 17

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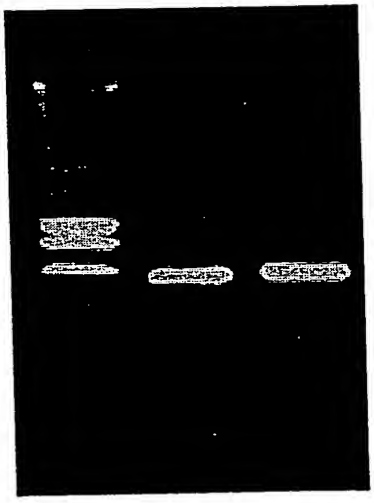


FIGURE 18

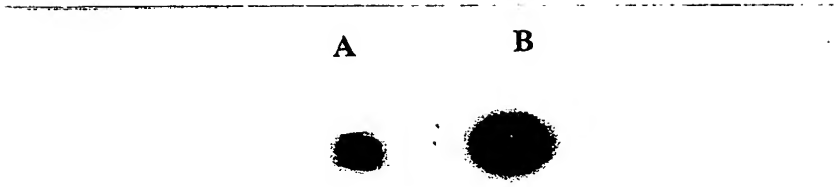
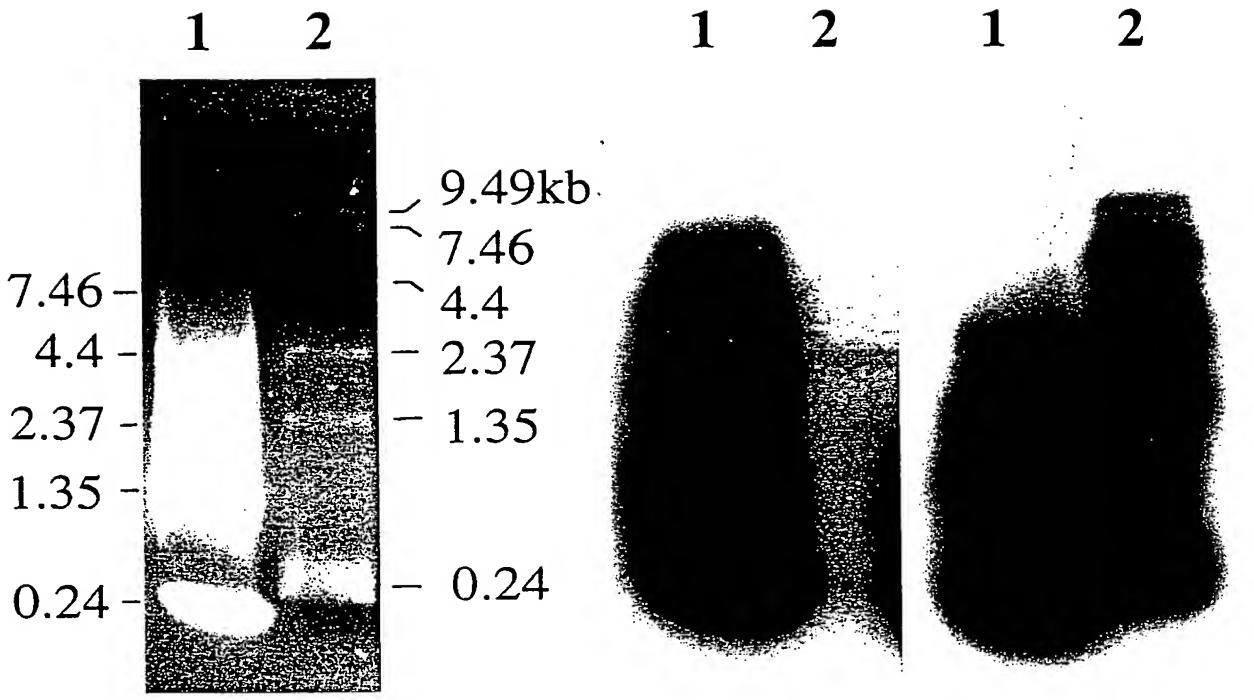


FIGURE 19



(Panel A) Agarose gel

(Panel B) Standard Northern

(Panel C) Deprotected RNA

FIGURE 20

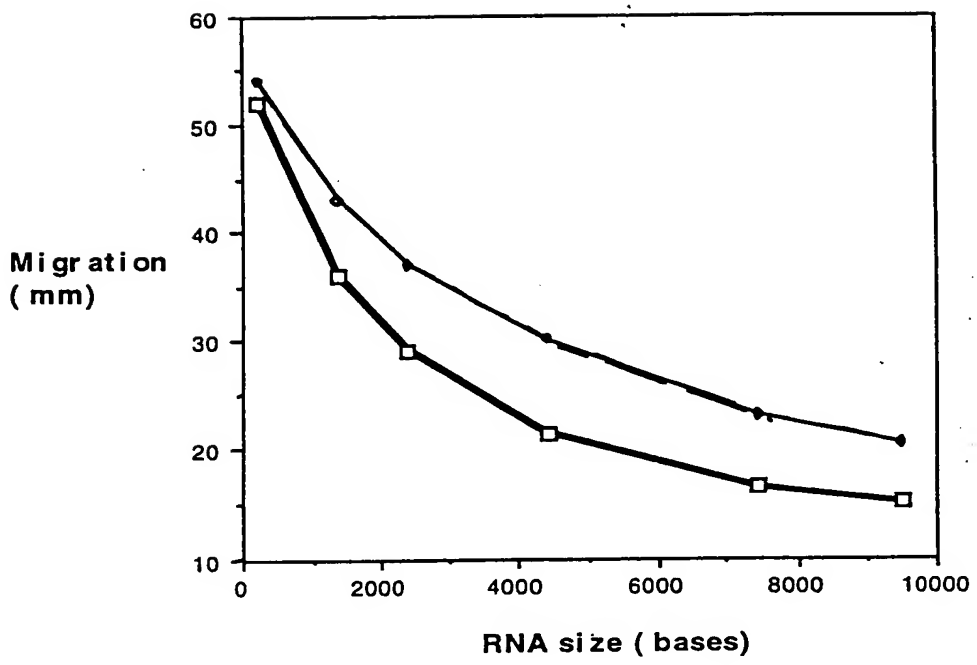

Migration
(mm)
60
50
40
30
20
10
0
2000
4000
6000
8000
10000
RNA size (bases)

FIGURE 21

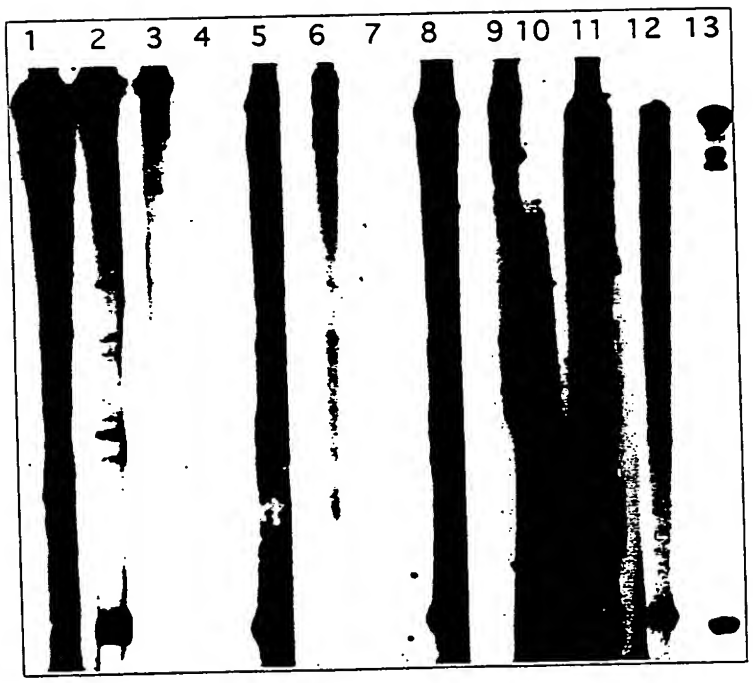


FIGURE 22

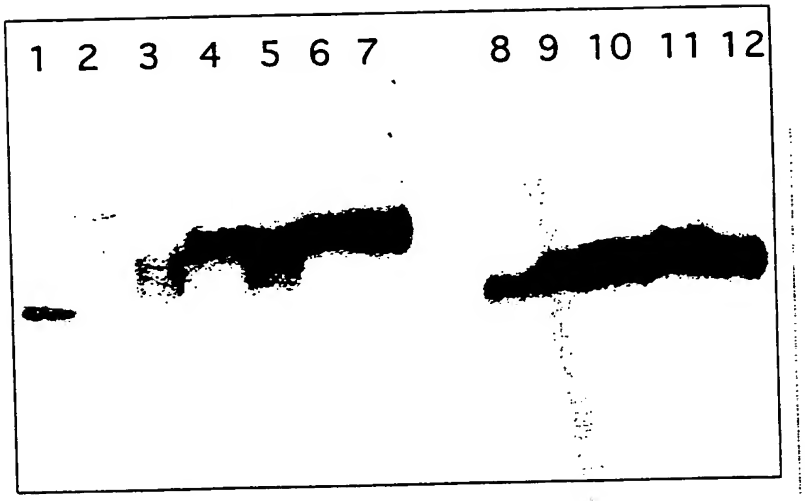


FIGURE 23

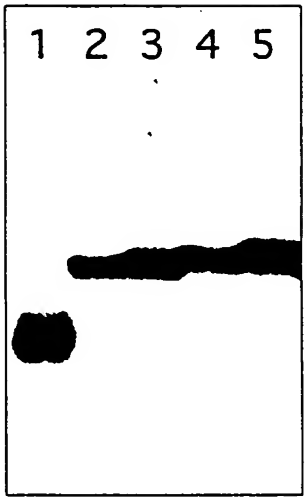


FIGURE 24

FIGURE 25

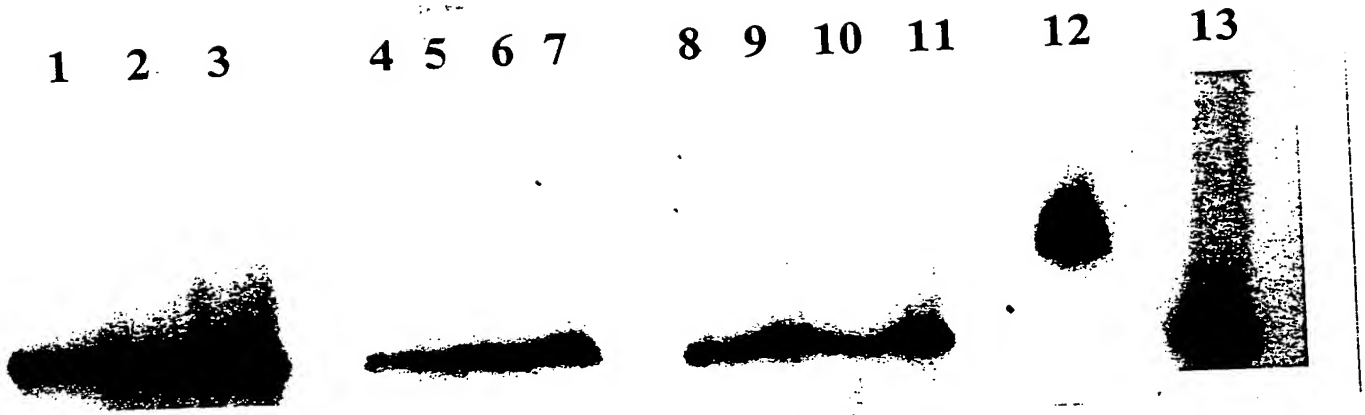


FIGURE 26